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제브라피시 배발생과정을 활용한 세포분열기작 연구

- Aurora-A 키나아제와 Kif18a 모터 단백질 기능 연구 -

Study on the mitotic machinery using zebrafish embryogenesis

- Defining the Role of Aurora-A kinase and Kif18a motor protein -

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서울대학교 대학원

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Study on the mitotic machinery using zebrafish embryogenesis

- Defining the Role of Aurora-A kinase and Kif18a motor protein -

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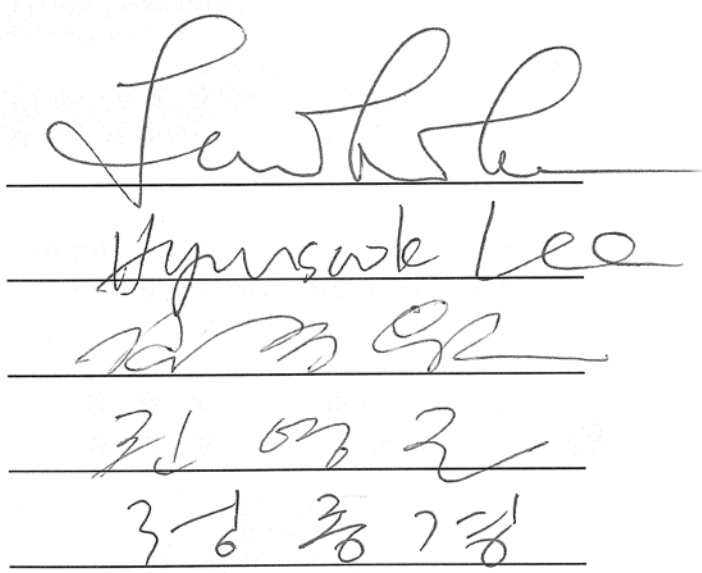

ABSTRACT

Study on the mitotic machinery using zebrafish embryogenesis

- Defining the Role of Aurora-A kinase and Kif18a motor protein -

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Cell division culminates with mitosis in which duplicated chromosomes are equally segregated to two daughter cells to maintain genomic integrity. To finely regulate the mitotic procedures, many mitotic kinases, phosphatases, and motor proteins are involved. In this study, I aim to discover the conserved

function of mitotic machineries such as Aurora-A kinase and Kif18a motor protein using zebrafish embryogenesis as a model system.

Aurora-A is a serine/threonine kinase, which regulates many intricate processes during mitosis in a phosphorylation-dependent manner. Despite the emerging interest for clinical applications, our understanding on Aurora-A function at the organism level is still limited. Here, I have discovered the function of Aurora-A in zebrafish embryo. Using morpholino (MO) technology, I showed that Aurora-A morphant zebrafishes were unhealthy displaying cell death and growth retardation with short and bended trunks. The developmental defects might be attributable to the abnormal mitotic progression, manifested by monopolar and/or disorganized spindle formation and mitotic arrest, accompanying spindle assembly checkpoint activation. Cell death in the absence of Aurora-A was partially rescued by p53 MO co-injection. Taken together, I elucidate that zebrafish Aurora-A is essential for mitotic progression and its depletion induces p53-dependent cell death, which can provide insight into the molecular mechanism of anti-cancer drug targeted for Aurora-A.

Chromosome movements in mitosis are directed by microtubules which are regulated by motor proteins. Kif18a is a kinesin-8 family motor protein, which has been identified as a suppressor of chromosome movements. It is known to regulate microtubule dynamics such as pre-anaphase chromosome oscillations and chromosome congression through two regulation modules: N-terminal catalytic motor domain and C-terminal tail domain. Tail domain in kinesin-8 is known to have controversies whether it has microtubule depolymerase activity or not. Despite of the important role of Kif18a in *in vitro* and cell-line data, there are still limited knowledge on the physiological role of Kif18a. Therefore, I attempted to dissect the role of two different Kif18a knockout zebrafishes: the C-terminus disrupted one using ZFN (zinc finger nuclease) technique and the motor targeted one using CRISPR/Cas9 technique. As Kif18a C-terminal domain is not required for the viability, fertility, and mitosis, I am now generating motor domain targeted Kif18a mutant zebrafish using a CRISPR/Cas9 knockout method. I suggest that this study, using zebrafish, provides the knowledge on the evolutionary conserved role and regulation mechanism of mitotic machineries.

Keywords: Zebrafish, embryogenesis, mitotic machinery, Aurora-A kinase, Kif18a motor protein

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I. INTRODUCTION

I-1. Cancer, mitosis and mitotic machinery

Cancer is a disease of genetic instability. Normal cells always can be threatened to be cancer cells because they are exposed to potential carcinogens such as ultraviolet lights, radioactive substances, and reactive species and also because they are going through the cell cycle. During the cell cycle progression, it is possible that cells have some errors in DNA replication and cell division. These erroneous events are mostly cured by innate devices, for example, checkpoints, apoptosis, and immune responses. But, if not, the cells have chance to be cancerous acquiring abilities to continuously divide. Hence, to understand, diagnose and treat cancer, characteristics of cancer cells need to be aware in terms of cell division.

Cell division culminates with mitosis, when duplicated chromosomes are equally segregated to two daughter cells to maintain genomic integrity. Mitosis is controlled by three main mechanisms which draw attention as druggable targets for anti-cancer therapies; 1) phosphorylation-dependent regulations, 2) ubiquitin-mediated proteasomal degradations, 3) motor protein activities (Jackson et al., 2007; Nigg, 2001; Peters, 2006; Rath and Kozielski, 2012; Walczak et al., 2010). These are connected each other in regulating mitosis and players such as mitotic kinases, phosphatases, proteasomes and motor proteins participate and fine-tune the elaborate and gorgeous mitotic movements.

For the phosphorylation-dependent regulation, many kinases and phosphatases exist. They are involved in the regulation of mitosis timely and spatially via localized in sub-cellular regions or spread in the cells. Cdk/cyclin complexes conduct the orchestra named as cell cycle, Cdk1/cyclin B is in charge of ‘mitosis’ part in metazoan. In addition to the conductor, there are other mitotic kinases such as Plk1 (polo-like kinase 1), Aurora-A, Aurora-B,

Mps1, etc. Plk1 has been known to have functions in the formation of spindle poles and microtubules and the establishment of KT-MT (kinetochore-microtubule) attachments. Aurora-A controls formation of spindle poles and astral microtubules and Aurora-B has been known as a component of CPC (chromosome passenger complex) to regulate KT-MT attachments and spindle assembly checkpoint activities (Ruchaud et al., 2007). Mps1 is known as an upstream kinase of SAC (spindle assembly checkpoint) signaling. PP1 or PP2A phosphatases are known to oppose those kinases, dephosphorylating and regulating various mitotic events such as microtubule dynamics and mitotic exits (Grallert et al., 2015).

After the use of mitotic players, proteasomal degradations occur to get rid of them. In mitosis, APC/C (anaphase promoting complex/cyclosome) is responsible for the protein degradation (Pines, 2011). APC/C has two co-factors, cdc20 and cdh1, to distinguish their substrates. When all chromosomes are attached to the microtubules and the SAC is satisfied, securin and cyclin B is degraded by APC-cdc20. In detail, securin constrains

separase to tighten pairs of duplicated chromosomes and to protect chromosomes from premature sister chromatid separation until the SAC is satisfied. And cyclin B is degraded when a cell exits mitosis. On the other hand, Aurora-A is degraded by APC/C-cdh1 after the mitotic exit.

Microtubule dynamics, which are driven by motor protein activities, regulate the locations of sub-cellular organelles and the movements of chromosomes (Verhey and Hammond, 2009). Eg5, kinesin-11 family protein, is involved in the separation of spindle poles and establishment of bipolar spindles. Kif18a, kinesin-8 family protein, negatively regulates chromosome oscillations.

The roles of mitotic players are so complicated that sometimes one molecule has multiple functions and sometimes several molecules are involved in the control of the same event. Moreover, the known functions are controversial among the systems used in the research and between species. That's why it needs to be studied the conserved functions of mitotic machineries in not-yet-proved *in vivo* model systems; conservation might mean the most ancient and

important function. Previous studies on mitotic machineries reveal that knockout mice for many of them, such as Cdk1, not interphase Cdks, Plk1, Aurora-A, etc., show embryonic lethality as they are important for mitosis (Lu et al., 2008a; Lu et al., 2008b; Santamaria et al., 2007). Also, incongruities on the conserved function of mitotic machineries have existed in the results from cell-lines and model organisms. Therefore, it is difficult to study the *in vivo* function of mitotic machineries. To circumvent early embryonic lethality and visualize mitosis in developmental stages, I adopted zebrafish model system. In this study, I aim to discover the conserved function of mitotic machineries such as Aurora-A kinase and Kif18a motor protein using zebrafish embryogenesis as model stages.

I-2. Zebrafish as a model for studying on mitotic machineries

Zebrafish is a vertebrate model that has many advantages to study on the function of mitotic machineries. It is easy to observe embryogenesis because

of *ex uterus* fertilization and transparent embryos, enabling the monitoring of cell proliferation and the physiological consequences of gene manipulation at the organism level. Also, as zebrafish lays hundreds of eggs in each clutch, there are almost unlimited numbers of eggs to inject morpholino (MO), RNA and DNA and to approach to genetics using transgenic, knockout or mutant zebrafish. Morpholino oligo-induced knockdown of gene expression in zebrafish embryogenesis is very efficient and does not interfere with maternal transcripts, and therefore, early embryonic lethality can be avoided.

Zebrafish has been used in a field of cancer and cell cycle since p53 mutant fish was generated (Berghmans et al., 2005) and recently, cell division in whole live embryos is trackable using Histone H2B-GFP transgenic fish (Jeong et al., 2010), NLS-Kikume and dual FUCCI system (Bouldin et al., 2014).

As previous studies on the mitotic machineries are mainly performed in the cell-lines and *in vitro* biochemical analysis in which are not enough to reflect actual physiological condition or mouse models in which show early

embryonic lethality. So, I propose that the function of mitotic machineries should be confirmed in various *in vivo* model system to reveal the truth and to reconcile discrepant results from model systems. Zebrafish embryogenesis is likely to reflect the cell proliferation of tumorigenesis, since rapid cell division cycle is one of the characteristics for the period of embryogenesis. Despite the existing limitations, it could be capable of adopting zebrafish embryos as a stages of in vivo drug discovery (Zon and Peterson, 2005) small molecule screening (Murphey and Zon, 2006) or drug validation.

I-3. Aurora-A mitotic kinase

Cdk/cyclin complexes are key regulators of cell cycle, but there are also other mitotic kinases like plks and Aurora families to finely regulate cell cycle in a phosphorylation-dependent manner. The first *aurora* allele was identified in a screen for *Drosophila melanogaster* mutants defective in spindle-pole behavior (Glover et al., 1995), which infers its roles associated in centrosomes

and proximal spindles. It was named after Aurora because of its resemblance to the aurora phenomenon of the night sky in the polar region. Aurora families are well-known Ser/Thr kinases regulating various mitotic events. Aurora-A is known to control centrosome maturation in some systems such as *Drosophila* sensory organ precursor cells and *C.elegans* (Berdnik and Knoblich, 2002; Hannak et al., 2001). Also, Aurora-A regulates mitotic entry at certain circumstances such as serum starvation and checkpoint recovery (Cowley et al., 2009; Macurek et al., 2008). The most critical role of Aurora-A is bipolar spindle formation and chromosome congression, which is driven by interaction with its cofactor TPX2 (Kufer et al., 2003; Kufer et al., 2002; Trieselmann et al., 2003) or phosphorylation of substrates such as TACC (Giet et al., 2002), Eg5 (Giet and Prigent, 2000; Giet et al., 1999), HURP (Yu et al., 2005) and CENP-E (Kim et al., 2010).

Aurora kinases have been attractive targets for novel anti-cancer therapeutics for many reasons. Human Aurora-A, located at chromosome 20q13.2, is commonly amplified in numerous malignant tumors including

breast, colon, bladder, ovary, skin and pancreatic cancers, and the levels of Aurora-A mRNA and protein are also increased in those tumors (Bischoff et al., 1998; Nikonova et al., 2012; Zhou et al., 1998). Furthermore, it is known that inhibition of Aurora-A induces cell death. Consequently, it is thought that inhibitors of Aurora kinases might be used as anticancer drugs (Keen and Taylor, 2004).

However, there are controversial results from different cell-lines or different organisms and the function of Aurora-A is debatable among experimental systems (Dutertre et al., 2002). Also there were disappointing results from clinical trials (Dees et al., 2011) such as disease progression despite of drug treatment or adverse effects, which indicate that there are more to learn about Aurora-A function at the organism level. Therefore, studying Aurora-A function in zebrafish system could provide valuable information. Furthermore, since the function of Aurora kinase in embryogenesis has not been fully understood yet because of the embryonic lethality (Jiang et al., 2008), zebrafish system could be useful in gene manipulation, such as

knockdown using MOs, and observance of the phenotypes in developmental stages in the presence of their maternal transcripts.

I demonstrate that the conserved Aurora-A has its role in mitosis and cell death during zebrafish embryogenesis by employing morpholino knockdown system. Aurora-A morphants exhibited problems in progression through mitosis, accompanying monopolar spindle formation and mitotic delay. There were significant increases of apoptosis, which was rescued by depleting p53. Taken together, these results have clinical implications in applying Aurora-A-specific inhibitors in the treatment of cancer.

I-4. Kif18a mitotic motor protein

Microtubules constantly polymerize and depolymerize, so-called “Microtubule dynamics”. Microtubule dynamics, which are regulated by motor proteins, are important for proper chromosome congression and

chromosome segregation. Among those motor proteins, Kif18a is a kinesin-8 family member protein, +end-directed motor, which have been identified as a suppressor of chromosome movements. It is known to regulate microtubule dynamics such as pre-anaphase chromosome oscillations and chromosome congression through two regulation modules: N-terminal catalytic motor domain and C-terminal tail domain that can have depolymerase activity. It is reported that the purified proteins from budding yeast kip3p (Varga et al., 2006) and human KIF18A (Mayr et al., 2007) depolymerize longer microtubules faster than shorter ones. Fission yeast homologs have no depolymerisation activity *in vitro* (Grissom et al., 2009), but they have been shown to promote catastrophes in a length-dependent manner *in vivo* (Tischer et al., 2009; Unsworth et al., 2008). Therefore, it is suggested that Kinesin-8s are not just modulators of microtubule dynamics, but may provide the negative feedback needed to control the length of microtubules. Contrary to the important role of Kif18a in *in vitro* and cell-line data, it is reported that Kif18a knockout/mutant mice only showed male infertility due to mitotic infidelity (Czechanski et al., 2015; Liu et al., 2010). This paradoxical results

remind questions; 1) what is the evolutionary conserved role of Kif18a and 2) whether the depolymerase activity of Kif18a is important or not in physiology? Here, I tried to dissect the role of Kif18a with two different knockout zebrafishes: C-terminus disrupted one using ZFN (zinc finger nuclease) technique and motor targeted one using CRISPR/Cas9. I show that C-terminus domain of Kif18a is dispensable for the viability and fertility of zebrafish as well as for the cell division, indicating motor domain is genuine functional part in development and mitosis.

II. MATERIALS AND METHODS

II-1. *Fish maintenance*

Wild-type zebrafish, *D. rerio*, were purchased from a local fish store and maintained in adequate facilities. Eggs were spawned and raised at 28.5 °C, injected and staged according to the standard procedures (Kimmel et al., 1995).

II-2. *Cloning of zebrafish Aurora-A, Mad2, and Kif18a*

Zebrafish Aurora-A, Mad2, Kif18a cDNAs were cloned by RT-PCR based on the mRNA sequence of ncbi databases (Aurora-A, GeneBank accession number NM_001003640.1; Mad2, NM_001017739.1; Kif18a, NM_200239.1). They were first cloned into pCR-BluntII-TOPO vector (Invitrogen), and then sub-cloned into pCS2+-EGFP vector.

II-3. *RNA extraction and RT-PCR*

Zebrafish embryos were collected along the stages, and the chorion were either poked (before bud stages) or dechorionated. RNA was extracted with TRIzol (invitrogen) as instructed in the manufacturer's protocols. Primers for RT-PCR and quantitative RT-PCR were used as following: Aurora-A forward; 5'-CTCCTCCAACACCAGTGGAT-3', Aurora-A reverse; 5'-GATGCTCCACTCCTGCTTTC-3', β-actin forward; 5'-CTCTTCCAGCCTTCCTTCCT-3', β-actin reverse; 5'-CTTCTGCATACGGTCAGCAA-3', bax forward; 5'-ACAGGGATGCTGAAGTGACC-3', bax reverse; 5'-GAAAAGCGCCACAACTCTTC-3', noxa forward; 5'-ATGGCGAAGAAAGAGCAAAC-3', noxa reverse; 5'-CGCTTCCCCTCCATTTGTAT-3', tp53 forward; 5'-TTGTCCCATATGAAGCACCA-3', tp53 reverse; 5'-ACACACACGCACCTCAAAAG-3', p21 forward; 5'-ATGAGGCTCAAATTGCTGCT-3', p21 reverse; 5'-

GGCTTTACGTGTGACCACCT-3'.

II-4. *In situ hybridization*

An antisense RNA probe was synthesized using SP6 RNA polymerase in the presence of DIG RNA labelling mix (Roche), and *in situ* hybridization was performed as described (Oxtoby and Jowett, 1993). Images were taken with Axioplan2 microscope using a 10X objective and the Axiovision software (Zeiss).

II-5. *Microinjection of mropholinos (MOs) and sense RNAs*

Translation-blocking Aurora-A ATG MO (5'-TGCTTCTTGCACCAGAGTCCATATC-3'), exon4-intron4 splicing-blocking Aurora-A MO (5'-ACAGCAGAAGTCCTCCTACCTGAGA-3'), Control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3'), p53 MO (5'-

GCGCCATTGCTTTGCAAGAATTG-3'), exon4-intron4 splicing-blocking Kif18a MO (5'-CACATGGAGGCAATTTTCACCTGGT-3') were designed and purchased from GeneTools (Philomath, OR). For RNA injection, Aurora-A and Mad2 cDNA were subcloned into pCS2-EGFP and then were transcribed *in vitro* using the mMessage mMachine kit (Ambion, Austin, TX). MOs or sense RNA were injected into the yolk of 1-cell to 8-cell stage embryos.

II-6. *Antibody generation in mice and western blotting*

GST-tagged zebrafish Aurora-A protein was purified and injected into mice peritoneum. Mice were sacrificed and the obtained serum was used for antibody against zebrafish Aurora-A. The serum was diluted at 5% BSA containing TBS/0.1% Tween-20 (1:200). α/β-Tubulin antibody (Cell signalling, 1:1,000) was used for loading control. HRP-conjugated secondary antibodies (Abcam) were diluted at 1:10,000.

II-7. *Immunofluorescence assay in whole embryos*

Embryos, collected, fixed in 4% paraformaldehyde and stored in cold methanol, were serially hydrated to PBS/0.1% Tween-20. Then, they were treated with cold acetone for 7 minutes at -20 °C and washed twice with PBS/0.1% Tween-20. After permeabilizing with PBS/1% DMSO/0.5% Triton-X100/0.1% Tween-20, embryos were incubated with blocking buffer containing 1% BSA, 10% goat serum in PBS/0.1% Tween-20. Primary and secondary antibodies were incubated in the blocking solution, washed intensively, and finally mounted in Vectashield with DAPI (Vector Laboratories). α-Tubulin antibody (DM1A, Sigma, 1:500), mouse and rabbit γ-Tubulin antibody (Sigma, 1:1,000), rabbit phospho-histone H3 (Ser10) antibody (pH3, Millipore, Billerica, MA, 1:1,000) were used as primary antibodies and Alexa fluro-conjugated secondary antibodies were used (1:200). Images of cells at the end of the trunk were taken and processed with Axio Observer Z1 (Zeiss) or DeltaVision (Applied Precision, WA) and then, processed and deconvolved using the SoftWorx program (Applied Precision,

WA).

II-8. *Flow cytometry*

During the prim-6 stage, the embryos were dechorionated, mechanically dissociated into single cells and fixed with 70% ethanol at -20°C. The cells were then immunostained with MPM-2, followed by 7-AAD (7-amino-actinomycin D) staining before flow cytometry analysis. Apoptotic cells were stained with *in situ* cell death detection kit, flurocein (Roche). Flow cytometry was performed using a FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA), as shown in the previous report (Jeong et al., 2010)

II-9. *Time-lapse microscopy of live embryos*

H2B-GFP transgenic zebrafish were used for the live-imaging and their

epithelial cells from the yolk surface area were taken for the film. Mitotic timing was measured as the duration between NEBD (nuclear envelop break down) and anaphase onset/mitotic exit. After the appropriate experiments such as morpholino injection, embryos were allowed to grow at 28.5 °C for 1 day and GFP-positive embryos were selected and placed on the chambered coverglass system (Nunc™ Lab-Tek™ II Chambered Coverglass) and embedded with 0.5% low-gelling agarose (Takara). Time-lapse microscopy was done on a DeltaVision. Optical sections (2 μm) of three consecutive images were collected every 2.5 min at 28.5 °C. Images were processed with SoftWorx or Image J software.

II-10. *Analysis of the spindle assembly checkpoint activation by Mad2 localization*

In vitro transcribed EGFP-zfMad2 mRNA was injected with MOs and then raised till prime-6 stage. Embryos were collected and dissociated as single

cells, and then, centrifuged at 1,200 rpm for 5 minutes in a Cytospin (Thermo Scientific). Cells, fixed with 4% paraformaldehyde, were immunostained with CREST (human nuclear ana-centromere autoantibody, Cortex Biochem) and mounted in Vectashield with DAPI. Images were taken by DeltaVision and processed, deconvolved and analyzed by SoftWorx program.

II-11. *TUNEL assay*

Whole mount *in situ* TUNEL assay was performed as described (Jeong et al., 2010). Images were taken by the same methods as *in situ* hybridization.

II-12. *Genotyping of Kif18a C-terminus deleted zebrafish*

Primers for wild-type allele in targeted exon 8 are as following: Forward; 5'-ATGTGATGAGCTTGGACAGTCA-3', Reverse; 5'-GGCAGCCCTCTTAGAATCTG-3', Primers for 5 nucleotides deleted

mutant allele are as following: Forward; 5’-AATGTGATGAGCTTGGACATAT-3’, Reverse; 5’-ACCCTGACCCTACAGTGCAA-3’. Melting temperature and cycle for PCR reaction is 56°C and 34 cycle.

III. RESULTS

Part 1. Depletion of Aurora-A in zebrafish causes growth retardation due to mitotic delay and p53-dependent cell death

III-1. Zebrafish Aurora-A is critically required for embryonic proliferation during development

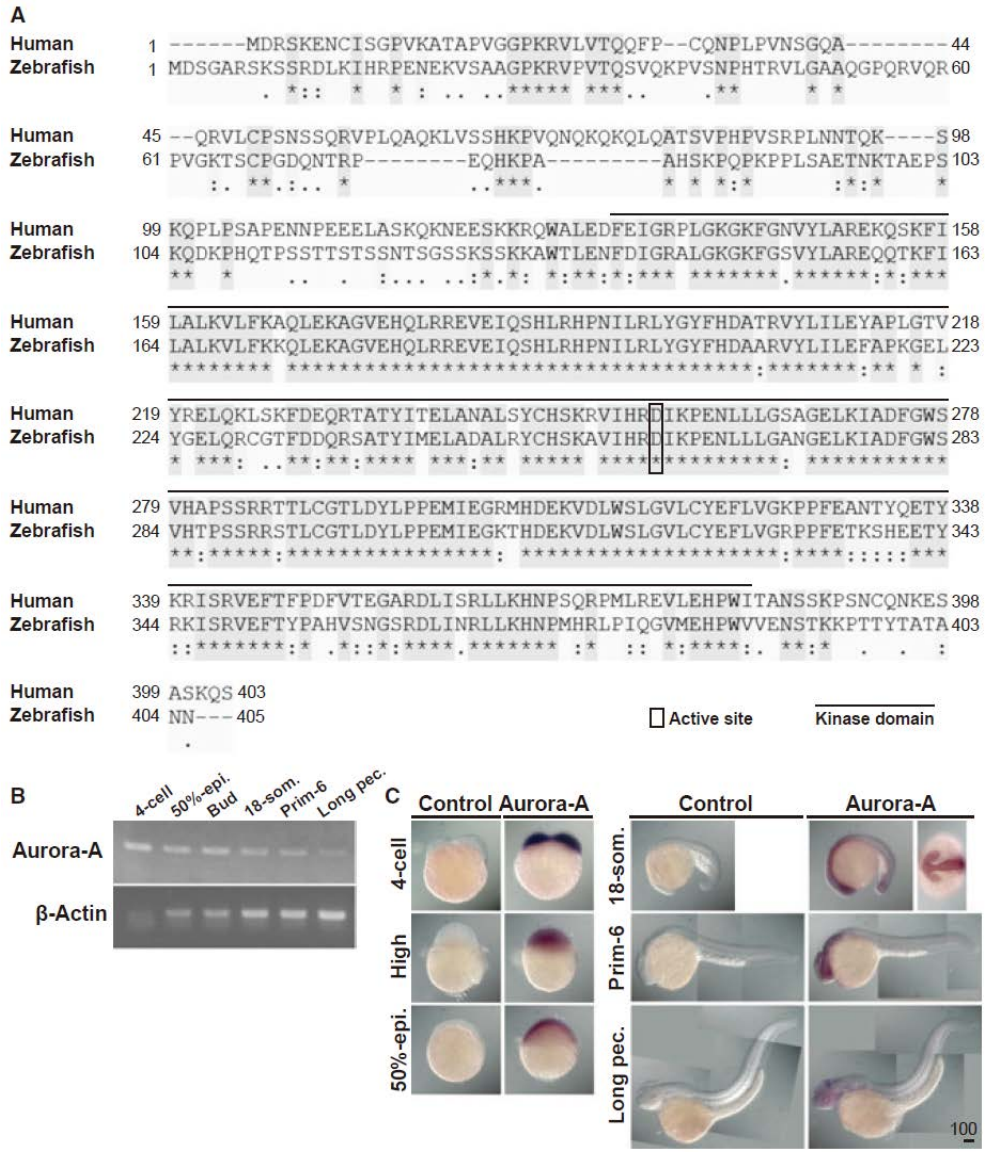
Putative Aurora-A in zebrafish (zgc: 100912) was found while searching for human Aurora-A orthologues using bioinformatics. It was designated as serine/threonine-protein kinase 6 with unknown function and located at zebrafish chromosome 6. When subjected to sequence alignment (Uniprot), it shows 56% of similarity in amino acid sequences. The kinase domain (amino acids 138–388 in zebrafish Aurora-A) was well conserved that it showed 94% similarity with human Aurora-A (Fig. 1A). The amino acid similarity of Aurora-A between *C. elegans*, *D. melanogaster*, *M. musculus*,

H. sapiens and *D. rerio* was ~33.3%.

To assess the expression level and pattern of zebrafish Aurora-A during developmental process, I performed RT-PCR and *in situ* hybridization assay. Zebrafish Aurora-A was expressed maternally and zygotically, as the mRNAs were detected from 4-cell stage embryos (Fig. 1B). *In situ* hybridization revealed that Aurora-A was expressed in all of the proliferating cells until the bud stage. From the 18-somite stage to the prim-6 stage, expression was abundant in the brain, tail, eyes, spinal cord, and somites, where cellular expansion took place during these embryonic stages (Fig. 1C). Both maternally and zygotically expressed Aurora-A mRNAs were ubiquitously expressed in actively dividing cells.

Figure 1. Aurora-A is conserved in zebrafish and its expression during embryogenesis suggests its role in development and cell proliferation. (A) Sequence alignment of Aurora-A in human and zebrafish, using Uniprot databases. The catalytic domain of human Aurora-A (line) and active site (box) are marked. Asterisks (*) mark the identical amino acid; colons (:) indicate the conservation between groups of strongly similar properties; a period (.) indicates conservation between groups of weakly similar properties. (B) RT-PCR analysis of Aurora-A expression during embryogenesis. β-actin was employed as the control. (C) *In situ* hybridization analysis of zebrafish Aurora-A. *Aurora-A* sense RNA was used for negative control. Images were taken on an Axioplan2 microscope using a 10X objective and the Axiovision software (Zeiss). Scale bar, 100 μm. Time post fertilization for the designated zebrafish developmental stages are as follows: 4-cell, 1 hour post fertilization (hpf); High, 3.3 hpf; 50%-epi., 5.3 hpf; 18-som, 18 hpf; Prim-6, 25 hpf; Long pec., 48 hpf.

Figure 1


A
Human 1 ------MDRSKENCISGPVKATAPVGGPKRVLVTQQFP--CQNPLPVNSGQA--------44
Zebrafish 1 MDSGARSKSSRDLKIHRPENEKVSAAGPKRVPVTQSVQKPVSNPHTRVLGAAQGPQRVQR 60
Human 45 --QRVLCPSNSSQRVPLQAQKLVSSHKPVQNQKQKQLQATSVPHPVSRPLNNTQK----S 98
Zebrafish 61 PVGKTSCPGDQNTRP--------EQHKPA---------AHSKPQPKPPLSAETNKTAEPS 103
Human 99 KQPLPSAPENNPEEELASKQKNEESKKRQWALEDFEIGRPLGKGKFGNVYLAREKQSKFI 158
Zebrafish 104 KQDKPHQTPSSTTSTSSNTSGSSKSSKKAWTLENFDIGRALGKGKFGSVYLAREQQTKFI 163
Human 159 LALKVLFKAQLEKAGVEHQLRREVEIQSHLRHPNILRLYGYFHDATRVYLILEYAPLGTV 218
Zebrafish 164 LALKVLFKKQLEKAGVEHQLRREVEIQSHLRHPNILRLYGYFHDAARVYLILEFAPKGEL 223
Human 219 YRELQKLSKFDEQRTATYITELANALSYCHSKRVIHRDIKPENLLLGSAGELKIADFGWS 278
Zebrafish 224 YGELQRCGTFDDQRSATYIMELADALRYCHSKAVIHRDIKPENLLLGANGELKIADFGWS 283
Human 279 VHAPSSRRTTLCGTLDYLPPEMIEGRMHDEKVDLWSLGVLCYEFLVGKPPFEANTYQETY 338
Zebrafish 284 VHTPSSRRSTLCGTLDYLPPEMIEGKTHDEKVDLWSLGVLCYEFLVGRPPFETKSHEETY 343
Human 339 KRISRVEFTFPDFVTEGARDLISRLLKHNPSQRPMLREVLEHPWITANSSKPSNCQNKES 398
Zebrafish 344 RKISRVEFTYPAHVSNGSRDLINRLLKHNPMHRLPIQGVMEHPWVVENSTKKPTTYTATA 403
Human 399 ASKQS 403
Zebrafish 404 NN--- 405
Active site
Kinase domain
B
4-cell
50%-epi.
Bud
18-som.
Prim-6
Long pec.
Aurora-A
β-Actin
C
Control
Aurora-A
4-cell
High
50%-epi.
18-som.
Prim-6
Long pec.
100

III-2. Knockdown expression of Aurora-A leads to growth retardation and cell death

Since our understanding about Aurora-A *in vivo* was still limited, functional studies of Aurora-A using zebrafish system could provide useful information. Therefore, to analyze the function of Aurora-A in zebrafish, I utilized morpholinos and validated the loss-of-function. I injected either Aurora-A translation blocking morpholinos or exon4/intron4 splice junction targeted splicing blocking morpholinos to 1-cell to 4-cell stage embryos. Here, Aurora-A MO, abbreviation for Aurora-A translation blocking morpholinos, were used for representative data. Upon injection of Aurora-A MO, morphant embryos showed morphological defects after the epiboly, the stages when MO started to work. Aurora-A morphants were unhealthy and showed growth defects including short, bended trunk, delayed development and cell death and these phenotypes were accumulated as time goes by (Fig. 2A). At 12-somite, the developments of brain and optic primordium were not well-established and the trunk was slightly shortened. At 18-somite, Aurora-A

morphants mainly showed cell death at brain region and at prim-6, 25 hpf (hour post fertilization), lengthening of somites and yolk extensions were defective because the width of the somites was reduced, not the number of the somites. And at long pec stages, 48 hpf, Aurora-A morphants had significantly smaller brain and eyes as well as a short and bended trunk.

As there were no commercial antibodies available for zebrafish Aurora-A, I made mouse antiserum against *E. coli* purified full-length Aurroa-A proteins. Western blot analysis with the home-made antibody raised against zebrafish Aurora-A indicated that Aurora-A was successfully depleted from the 3-somite stage (Fig. 2B). Both Aurora-A ATG MO and the splice MO similarly depleted Aurora-A (Figure 3A). This result is consistent with the notion that the outcome of ATG MO- and splice MO-injection were similar, indicating the phenotypes are caused by Aurora-A depletion, not by morpholino toxicity. Morpholinos are relatively less effective towards the abundant maternal RNA and proteins. Therefore, it is not surprising that Aurora-A expression is not reduced until the 50%-epiboly stage. When the overall survival was measured,

Aurora-A morphants (both Aurora-A MO and splicing MO) exhibited deaths from 4 dpf (days post fertilization) with an accumulated defects and only 40 % of embryos survived in 6 dpf (Fig. 3B).

Figure 2. Aurora-A morphants display growth defects and cell death. (A) Comparison of control and Aurora-A morphants from 4-cell stage embryos to the long pectoral fin stage. Arrows point to the apparent growth defects. Images of live embryos immobilized on 3% methylcellulose were obtained with Axio Imager A1 (4X objective). Scale bar, 100 μm. (B) Efficiency of knockdown expression by the Aurora-A MO injection with Western analysis (WB). Home-made anti-Aurora-A serum (mouse polyclonal) detects ~50 kDa band. Aurora-A disappears upon MO injection from the 3-somite stage, indicating that morpholino injection does not abolish maternal proteins. Extracts from 4 embryos were loaded per lane. Time post fertilization: High, 3.3 hpf; 50%-epi, 5.3 hpf; 3-som, 11 hpf; 12-som, 15 hpf; 18-som, 18 hpf, Prim-6, 25 hpf; Long pec., 48 hpf.

Figure 2

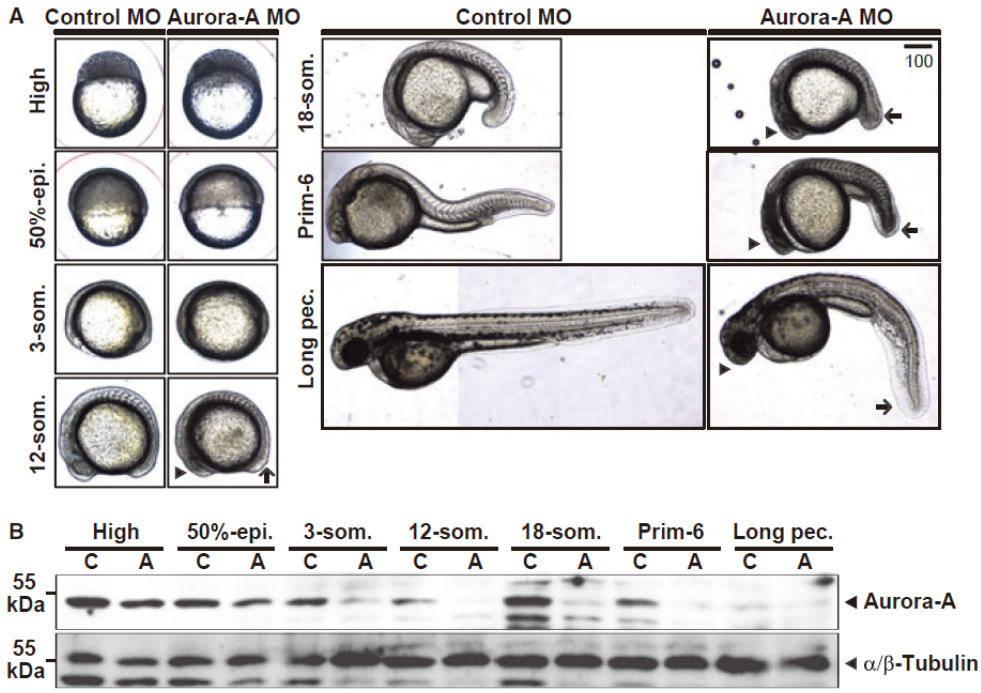
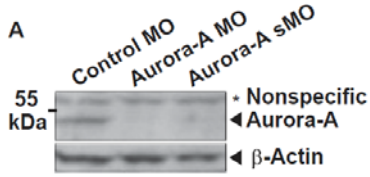

A
Control MO
Aurora-A MO
Control MO
Aurora-A MO
High
50%-epi.
3-som.
12-som.
18-som.
Prim-6
Long pec.
100
B
High
50%-epi.
3-som.
12-som.
18-som.
Prim-6
Long pec.
C A C A C A C A C A C A C A
55 kDa
55 kDa
Aurora-A
α/β-Tubulin

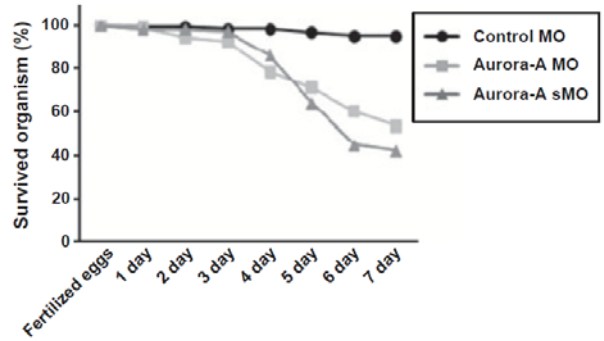
Figure 3. Two kinds of morpholinos support that the death of cell and embryo in Aurora-A morphants is a genuine phenotype, not from the morpholino toxicity. (A) Aurora-A MO (ATG MO) and Aurora-A sMO (exon4-intron4 splicing blocking MO) were injected to embryos and western blot analysis was performed at 1 dpf, using 4 embryos. (B) Viability of embryos injected with control MO, Aurora-A ATG MO, Aurora-A splicing MO. Numbers of live embryos were counted every day. Viability of the injected embryos is shown as a survival graph. Injection of two different types of morpholino induces similar survival tendency that supports the death phenotype in Aurora-A morphants is a genuine phenotype, not caused by the morpholino toxicity.

Figure 3



B

	Fertilized eggs	1 day	2 day	3 day	4 day	5 day	6 day	7 day
Control MO	122	121	121	120	120	118	116	116
Aurora-A MO	108	107	102	100	85	77	65	58
Aurora-A sMO	109	107	107	106	94	70	49	46



III-3. Knockdown of Aurora-A induces defects in mitotic progression

As the defective phenotype from Aurora-A morphants were very interesting and inspiring, I began to think about what actually caused these defective phenotypes. First, I assessed the problem in the mitotic progression by staining the embryos with phospho-histone H3 (pH3), the marker for late G2 and early mitosis (Hendzel et al., 1997). Consistent with the previous report from mouse system (Cowley et al., 2009), depletion of Aurora-A in zebrafish embryos led to increase of pH3-positive cells. This result was partially rescued by Aurora-A-EGFP mRNA co-injection, confirming that the mitotic arrest was due to knockdown of Aurora-A (Fig. 4A).

Next, I asked whether the mitotic effect was due to delay in progression to anaphase by staining with MPM-2 monoclonal antibody which detects phospho-epitopes in mitosis (Davis et al., 1983). Previously, it is suggested MPM-2 staining as the useful index for assessing spindle assembly checkpoint activation (Choi et al., 2009; Lee et al., 1999). MPM-2 positive cells were increased to 5-fold (Fig. 4B) in Aurora-A morphants and it was

confirmed by more than 3 other independent experiments. These results implied that mitotic progression defects in Aurora-A morphants might be due to the activation of spindle assembly checkpoint.

To analyze the basis of SAC activation and mitotic arrest after Aurora-A depletion, embryos were collected at prim-6 stages, immunostained with anti-α-tubulin and anti-γ-tubulin antibodies, and subjected to immunofluorescence analysis. In mitosis, 67.4% of Aurora-A morphant cells exhibited abnormal spindle organization (n=62, Figure 5A): 47.8% (n=44) exhibited monopolar spindles (Fig. 5A, prometaphase, Aurora-A MO, 1st and 2nd rows); 19.6% (n=18) displayed disorganized spindles with two centrosomes (Fig. 5A, prometaphase, Aurora-A MO, 3rd row). There were cells segregating chromosomes with two centrosome in Aurora-A morphants, but in the γ-tubulin immunofluorescence intensity was lower compared to control (Fig. 5A, metaphase and Fig. 6). Only 5.7% of mitotic cells (3 out of 52 cells) displayed abnormal spindles in the controls. When EGFP-Aurora-A mRNA was injected together with Aurora-A MO, it partially rescued the phenotype

that bipolar cells increased. EGFP-Aurora-A co-localized with the γ-tubulin, indicating that Aurora-A mainly localizes to the centrosomes (Fig. 5B). From above result, it is noticeable that mitotic progression is defective in the absence of Aurora-A. Thus, I could conclude that Aurora-A is a key molecule for centrosome maturation and spindle organization in zebrafish embryogenesis, hence involved in appropriate chromosome congression. These results altogether suggest that zebrafish Aurora-A is required for the intactness of centrosome to establish bipolar spindle assembly.

Figure 4. Knockdown of Aurora-A induces defects in mitotic progression.

(A) Immunostaining with anti-phospho-histone H3 antibody in control, Aurora-A morphant, Aurora-A MO and EGFP-Aurora-A RNA co-injected. pH3-positive cells are marked with red and DNA was counter-stained with DAPI (4', 6'-diamidino-2-phenylindole). Images were taken with Axio Observer Z1 with a 20X objective. Scale bar, 50 μm. (B) Flow cytometric analysis after MPM-2 immunostaining and 7-amino actomycin D (7-AAD). 2C and 4C DNA contents measured by 7-AAD staining are marked. MPM-2-positive 4C DNA contents in the box are the cells arrested in mitosis.

Figure 4

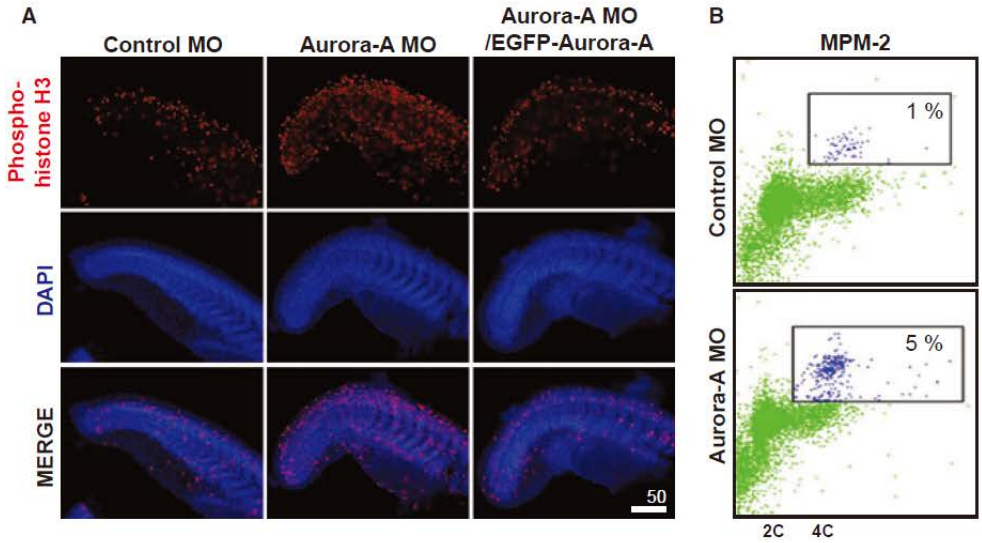

A
Control MO
Aurora-A MO
Aurora-A MO
/EGFP-Aurora-A
Phospho-
histone H3
DAPI
MERGE
50
B
MPM-2
Control MO
1 %
Aurora-A MO
5 %
2C
4C

Figure 5. Knockdown of Aurora-A induces abnormal spindle formation.

(A) Immunofluorescence assay with anti-α-tubulin and anti-γ-tubulin antibodies in fixed embryos at 25 hpf after control or Aurora-A MO injection. Representative prometaphase and metaphase cells are shown in each group. α-tubulin is shown in red, γ-tubulin in green and DNA in blue (counterstained with DAPI). (B) Co-injection of *EGFP-Aurora-A* with control or Aurora-A MO. Expression of *EGFP-Aurora-A* shows its subcellular localization at centrosomes with γ-tubulin. Moreover, expression of *EGFP-Aurora-A* in Aurora-A MO rescues spindle abnormalities, as it forms relatively normal bipolar spindles. Images were acquired, processed, and deconvoluted with DeltaVision (100X objective) and the SoftWorx program (Applied Precision). Scale bar, 5 μm.

Figure 5

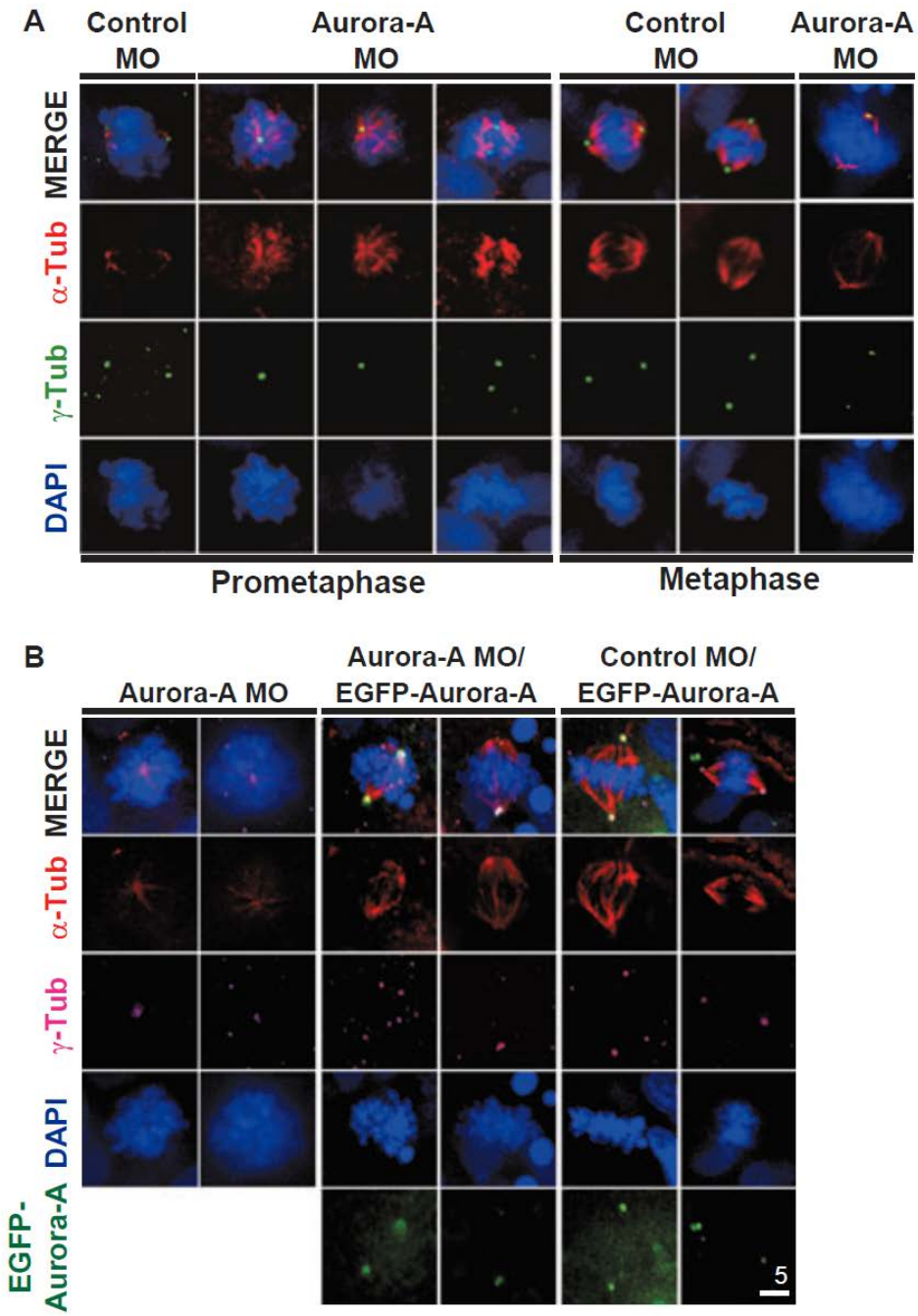
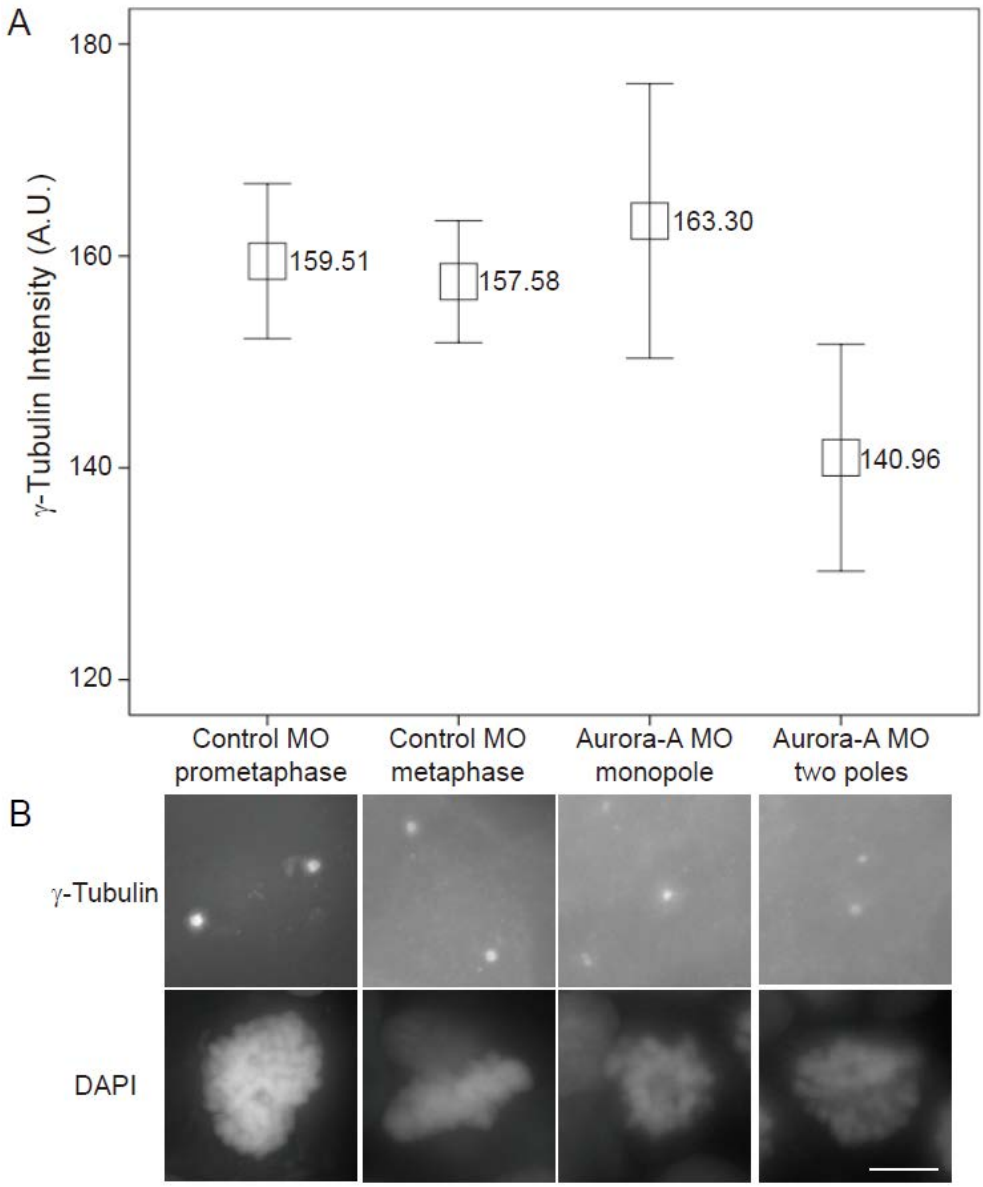

A
Control MO
Aurora-A MO
Control MO
Aurora-A MO
MERGE
α-Tub
γ-Tub
DAPI
Prometaphase
Metaphase
B
Aurora-A MO
Aurora-A MO/ EGFP-Aurora-A
Control MO/ EGFP-Aurora-A
MERGE
α-Tub
γ-Tub
DAPI
EGFP-Aurora-A
5

Figure 6. Aurora-A morphants show problems in centrosome intactness, such as centrosome maturation. (A) Problems in centrosome intactness, such as centrosome maturation, of Aurora-A morphants. I quantified the centrosome intactness by immunostained γ-tubulin fluorescence under same exposure, using Image J software. More than 12 cells were scored. MEAN ± S.E.M.: Control MO prometaphase, 159.51± 4.63; Control MO metaphase, 157.58 ± 4.77; Aurora-A MO monopole, 163.30±12.97; Aurora-A MO two poles, 140.96 ± 6.69. Data were analyzed with SPSS software. (B) Representative image of (A). In control cells, average γ-tubulin intensity was similar in prometaphase and metaphase cells. In Aurora-A morphants, monopoles have slightly stronger intensity than control. Two poles in Aurora-A morphants have weaker intensity than control. Scale bar, 5μm.

Figure 6


A
180
160
140
120
γ-Tubulin Intensity (A.U.)
159.51
157.58
163.30
140.96
Control MO prometaphase
Control MO metaphase
Aurora-A MO monopole
Aurora-A MO two poles
B
γ-Tubulin
DAPI

III-4. Aurora-A morphants show delayed mitotic timing which may be due to the SAC (spindle assembly checkpoint) activation

I utilized Histone H2B-GFP transgenic zebrafish and carried out time-lapse microscopy in order to analyze the fate of individual cells after blocking zebrafish Aurora-A (Fig. 7B-D).

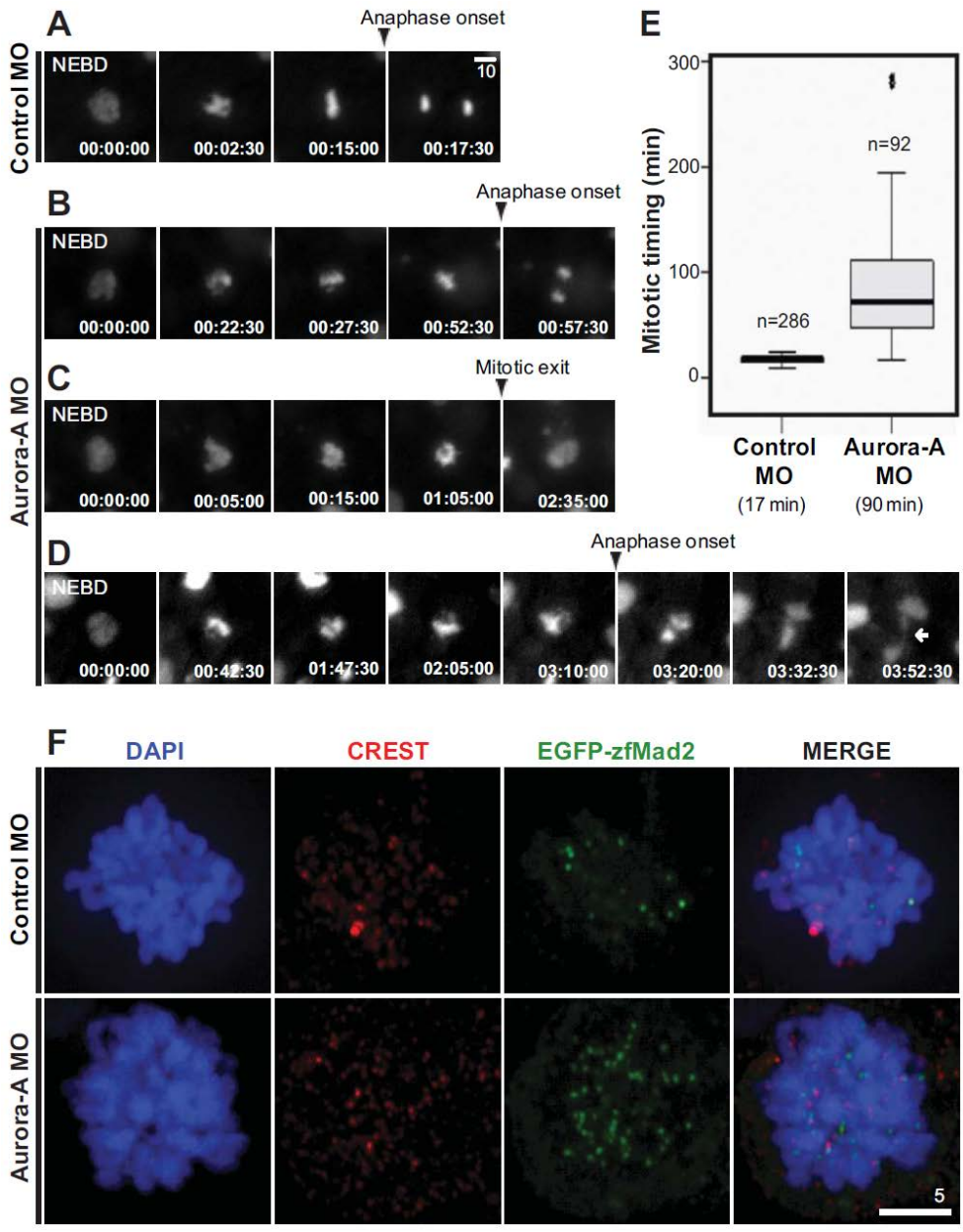
Live-images were taken and cell divisions were observed from 92 cells of 6 morphant embryos. The time from NEBD (nuclear envelope break down) to anaphase onset or decondensation was termed mitotic timing. All cells from Aurora-A morphants displayed delayed mitotic timing: more than 5-fold compared to control. In control, mitotic timing was 15-18 minutes; however, in Aurora-A depleted cells, the mitotic timing was 90 minutes on average (Fig. 7E). In Aurora-A morphants, chromosomes moved back and forth due to congression failure and got arrested in prometaphase after the NEBD. And 91.3% (n=92) of cell was eventually divided after the long arrest. Occasionally, some cells (4.35%, n=92) could not divide and decondensed, the mitotic exit without division. This phenomenon is consistent the cells

from Aurora-A knock-out mice (Cowley et al., 2009). Some cells (6.52%, n=92) showed misaligned chromosomes and/or lagging chromosomes. Only 2 cells (2.17%, n=92) showed cell death within mitosis.

I asked whether Aurora-A depletion induced SAC (spindle assembly checkpoint) activation. EGFP-zfMad2 mRNA was injected with MOs and 24 hpf embyos were subsequently dissociated into single cells and stained with CREST by immunocytochemistry methods. In Aurora-A morphant cells, localization of zfMad2 increased 4.6-fold compared to that of control cells (Fig. 7F).

Figure 7. Aurora-A morphants show delayed mitotic timing which may be due to the SAC activation. (A) Zebrafish cell division was monitored with high resolution time-lapse microscopy. Epithelial cells of yolk surface region were taken. Cells of control morphants took 15-18 minutes of mitotic timing, from NEBD to anaphase onset. (B-C) Cells in Aurora-A morphants showed significant delay in mitotic timing (5-fold increased 90 minutes on averages). Cells were arrested in prometaphase, and chromosomes kept moving before segregation (84/92, 91.3%). (D) Some cells could not divide after NEBD and decondensed (4/92, 4.35%), which is referred as mitotic exit without division. Scale bar, 10 μm. (E) Mitotic timing was measured and shown in the box plot. (F) Mitotic delay in Aurora-A morphants might be due to SAC activation. EGFP-zfMad2 were co-injected with morpholinos, and immunostained CREST were shown with red dots. Mad2 that co-localized with CREST was increased in Aurora-A morphants. Images were taken, processed and deconvolved with DeltaVision (100X objectives) and SoftWorx program (Applied Precesion). Scale bar, 5 μm.

Figure 7


A
Anaphase onset
Control MO
NEBD
10
00:00:00
00:02:30
00:15:00
00:17:30
B
Anaphase onset
NEBD
00:00:00
00:22:30
00:27:30
00:52:30
00:57:30
C
Mitotic exit
Aurora-A MO
NEBD
00:00:00
00:05:00
00:15:00
01:05:00
02:35:00
D
Anaphase onset
NEBD
00:00:00
00:42:30
01:47:30
02:05:00
03:10:00
03:20:00
03:32:30
03:52:30
E
Mitotic timing (min)
300
200
100
0
n=92
n=286
Control MO
(17 min)
Aurora-A MO
(90 min)
F
DAPI
CREST
EGFP-zfMad2
MERGE
Control MO
Aurora-A MO
5

III-5. Cell death in Aurora-A morphant embryos is p53-dependent

When Aurora-A MOs were injected, embryos showed developmental abnormalities such as bended trunk partly due to proliferation failure (Fig. 2A). I asked whether there was apoptosis after Aurora-A depletion using color-labeled whole-mount TUNEL assay (Fig. 8). I found that Aurora-A morphants underwent apoptosis upon Aurora-A depletion. At 3-somite stages embryos, about 3 hours after effective knockdown of Aurora-A, the number of apoptotic cells was relatively small amount, but it increased with each subsequent stage. Apoptotic cells were observed across whole embryonic body at 12-somite, when primordial developments of head and tail were balanced. The head region especially showed apoptosis at 18-somite, the stage when distinctive brain and eyes form. During prime-6 stages, when trunk and tail elongate and yolk extension gets lengthened, apoptosis occurred at trunk and tail region. Thus, those results suggest that apoptosis took place at the proliferating cells. Cell death as a consequence of Aurora-A depletion may be explained by three possible mechanisms: p53-dependent post-mitotic

apoptosis, unknown interphase effect before mitosis, and mitotic cell death. However, in live-imaging, the cells in Aurora-A morphants entered mitosis in normal manner (Fig. 7) compare to control. Additionally, only 2 out of 92 cells were found to exhibit death in mitosis.

I asked whether the apoptosis was p53-dependent. Aurora-A/p53 double MO experiments were performed (Fig. 9). MOs were injected in combination of control MO, Aurora-A MO and p53 MO and then isolated to single cells and prepared for FACS analysis at 24 hpf stages. Sub-G1 population was increased from 5.9 % to 17.7% in Aurora-A MO injected group. And it was reduced by p53 co-injection to 7.8 %. p53 did not affect the mitotic delay, as the level of MPM-2-positve cells in Aurora-A single morphants and Aurora-A/p53 double morphants were similar. With color-labeled TUNEL assay, apoptotic cells were stained in whole embryos. Apoptosis phenotype in Aurora-A morphants were partially rescued in Aurora-A/p53 double morphants. Whereas, in Aurora-A/p53 double morphants, there weren't any other significant morphological defects except for the effect upon Aurora-A

depletion.

I double-checked p53-dependent apoptosis upon Aurora-A depletion with quantitative real-time RT-PCR analysis (Fig. 10). Embryos collected at 24 hpf stages were used for the analysis and the relative mRNA expression levels of *Bax* and *Noxa*, apoptotic target genes of p53, were increased in Aurora-A morphants compared to that of *P21*, a cell cycle related target gene. Collectively, Aurora-A depletion induces mitotic arrest, following by cell death in subsequent cell cycle stages, which is p53-dependent.

Figure 8. Aurora-A knockdown induces apopototic cell death. Whole mount *in situ* TUNEL assay to analyze apoptosis in control and Aurora-A morphants. At 3-somite stages embryos the number of apoptotic cells was relatively small amount, but it increased with each subsequent stage. Apoptotic cells were observed across whole embryonic body at 12-somite, when primordial developments of head and tail were balanced. The head region especially showed apoptosis at 18-somite, the stage when distinctive brain and eyes form. During prime-6 stages, when trunk and tail elongate and yolk extension gets lengthened, apoptosis occurred at trunk and tail region. Thus, those results suggest that apoptosis took place at the proliferating cells. Scale bar, 100 μm.

Figure 8

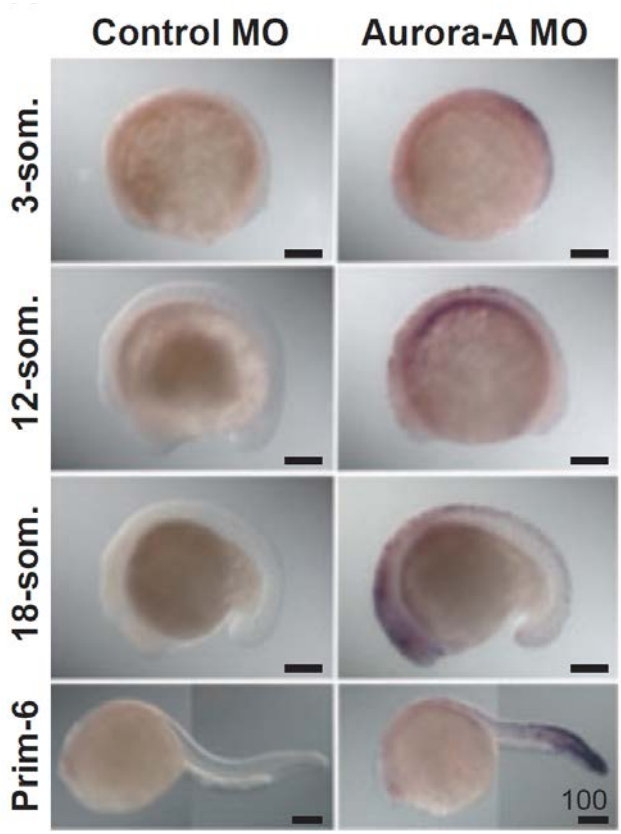
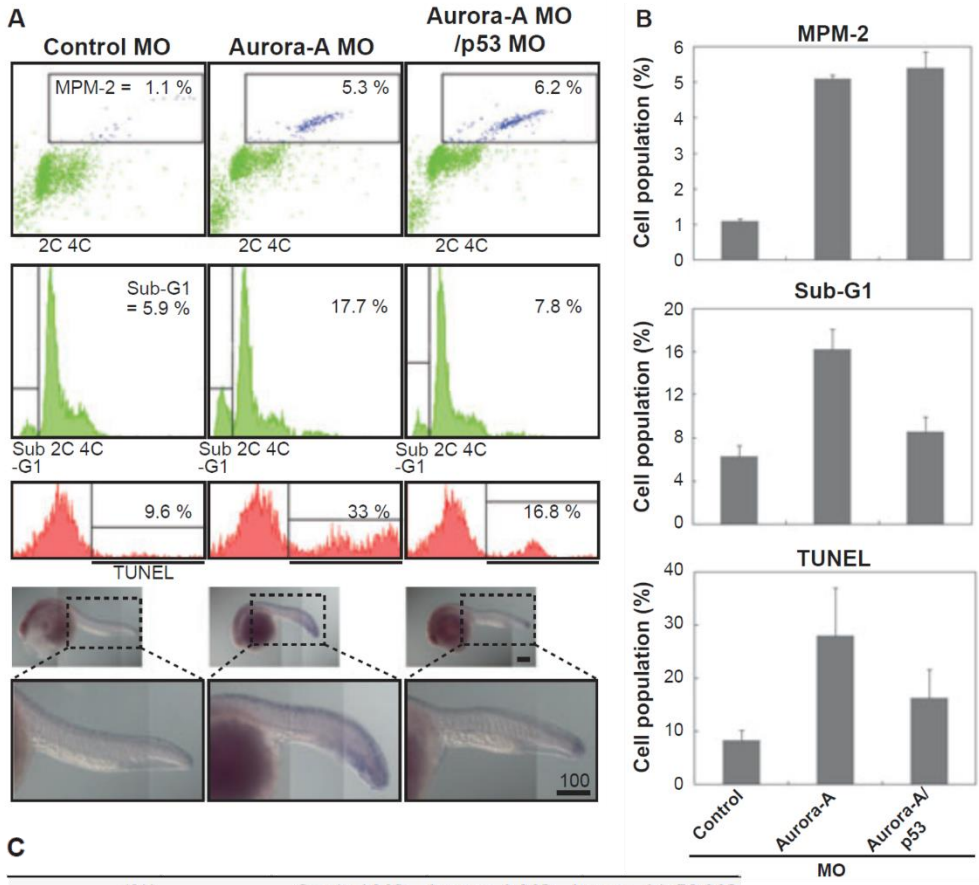


Figure 9. p53-dependent apoptosis is induced by Aurora-A depletion. (A) Representative flow cytometry analysis in control, Aurora-A morphant, and Aurora-A MO and p53 MO double injected embryos. MPM-2 positive cell population are marked and shown on top. Sub-G1 population (bottom left population) in 7-AAD staining marks apoptotic cells (middle). TUNEL positive cells presented by histograms are shown in the third row. The numbers mark the percentage of cells measured by flow cytometry. It increases markedly in Aurora-A MO and is decreased in Aurora-A MO/p53 MO double-injected embryos. The color-labeled TUNEL assay and its enlarged images are shown at the bottom. Images were taken with an Axioplan2 (10X objective). Scale bar, 100 μm. (B) Summary of three independent experiments. Error bars are MEAN+S.E.M. (C) Three independent experiments. Percentage of cells in sub G1, MPM-2-positive, and TUNEL-positive cells are shown in a table. For a single flow cytometry analysis, 20,000 cells from more than 50 embryos per each experimental group were used. Experiment 1 is the representative data.

Figure 9

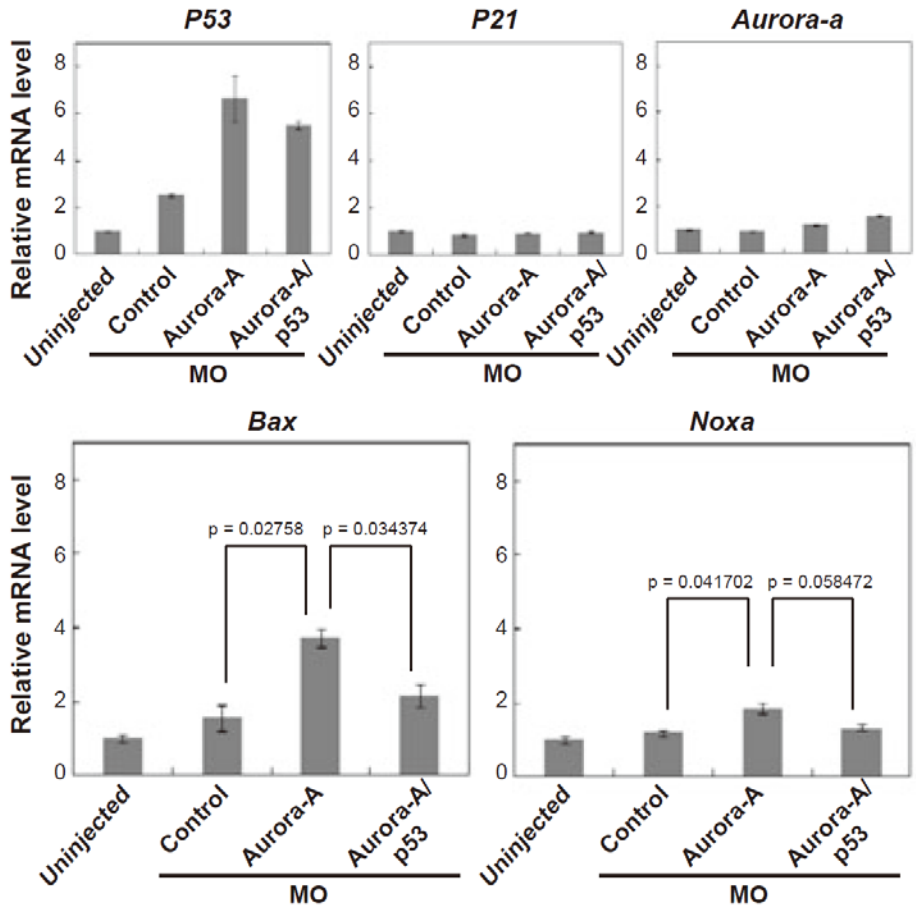


C

(%)		Control MO	Aurora-A MO	Aurora-A/p53 MO
Experiment 1	MPM-2	1.1	5.3	6.2
	Sub-G1	5.9	17.7	7.8
	TUNEL	9.6	33	16.8
Experiment 2	MPM-2	1	5	5.4
	Sub-G1	4.9	12.5	6.9
	TUNEL	4.6	10.4	6.8
Experiment 3	MPM-2	1.2	5	4.6
	Sub-G1	8.2	18.4	11.2
	TUNEL	10.7	40.5	25.2

Figure 10. Aurora-A depletion induces expression of pro-apoptotic downstream targets of p53, not the cell cycle related target gene. (A) Quantitative RT-PCR to analyze the indicated mRNA levels in control, Aurora-A MO-injected, and Aurora-A MO/p53 MO double-injected embryos. Relative expression level of mRNA was normalized to *β-actin*. Aurora-A depletion induces *p53* expression, which might induce two different target pathways, expression of *p21* to induce interphase cell cycle block or expression of pro-apoptotic genes leading to cell death. Aurora-A depletion does not induce *p21* expression, the cell cycle related target gene, but markedly increases the expression level of apoptotic target genes, *bax* and *noxa*. Results are from three independent experiments. Error bar, MEAN ± S.E.M. *P values* are marked.

Figure 10


P53
P21
Aurora-a
Relative mRNA level
0
2
4
6
8
Uninjected
Control
Aurora-A
Aurora-A/ p53
MO
Bax
Noxa
p = 0.02758
p = 0.034374
p = 0.041702
p = 0.058472

Part 2. Dissecting the role of Kif18a in zebrafish: whether Kif18a is a motor or a depolymerase

III-6. Zebrafish Kif18a is conserved and its expression pattern suggests its importance during embryogenesis

Kinesin motor proteins basically have four functional domains: Microtubule motor head, which is important to walk along the microtubules powered by ATP hydrolysis, the neck and stalk domain providing the flexibilities and dimerization scaffolds, and tail domain where cargos bind. There are some reports that Kif18a has microtubule depolymerase activity in their C-terminal tail domain.

At the beginning, I sub-divided zebrafish Kif18a into the four domains according to amino acid sequence homology with human Kif18a to check whether zebrafish Kif18a is evolutionarily conserved and which domain is the most significant. When subjected to amino acid sequence alignment,

Kif18a motor domain is well-conserved between human and zebrafish (Fig. 11).

To provide the clue for the role of Kinesin-8 family in zebrafish embryogenesis, I checked mRNA expression pattern using RT-PCR and *in situ* hybridization assay. Zebrafish has two kinesin-8 family proteins, Kif18a and Kif19, and they are expressed as both maternal and zygotic transcripts. Maternal to zygotic transition (MZT) seems to occur at 3 hpf (hour post fertilization) for zKif18a, 3-6 hpf for zKif19. And Kif19 expression upregulated at 6 days suggests that Kif18a is more important in early embryogenesis but there may exist compensation mechanism between Kinesin-8 family proteins along developmental stages (Fig 12A).

Besides, Kif18a mRNAs are mostly expressed where mitosis takes place such as cells in cleavage and blastula stages, heads and trunk according to the results from *in situ* hybridization assay (Fig 12B).

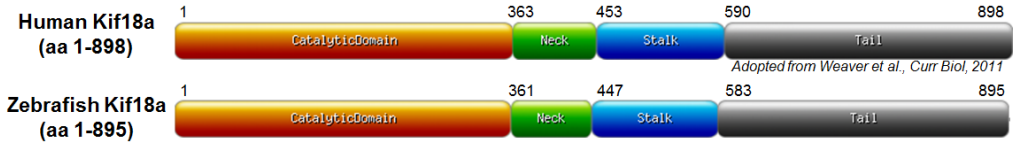
Taken together, these results suggest that the Kif18a might have important

roles in mitosis and development in zebrafish. Previously reported discrepant results on the Kif18a between cell-lines and mice could be reconciled and actual conserved roles of Kif18a could be revealed with the results from zebrafish.

On the other hand, there is a report that Kif18a mRNA is expressed in mouse embryonic brain as it shows by northern blotting (Miki et al., 2001). As Kif18a mRNA is expressed embryonic brain in zebrafish in my result, it suggests that Kif18a could be involved in neurodevelopment.

Figure 11. Kif18a is well-conserved between human and zebrafish. Human and zebrafish Kif18a domains are depicted. Kinesin motor proteins basically have four functional domains: Microtubule motor head, which is important to walk along the microtubules powered by ATP hydrolysis, the neck and stalk domain providing the flexibilities and dimerization scaffolds, and cargo binding tail domain. The domains for zebrafish Kif18a are sub-divided according to the amino acid sequence homology with human Kif18a (Weaver et al., 2011). Amino acid sequences are aligned by ClustalW2. Kif18a motor domain is well-conserved between human and zebrafish.

Figure 11



	Full Length	Catalytic Domain	Neck	Stalk	Tail
Identity	46.10%	72.92%	41.11%	41.60%	19.70%
Similarity	56.06%	82.04%	48.88%	52.55%	31.04%

Aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and Analyzed by SIAS (http://imed.med.ucm.es/Tools/sias.html)

Catalytic Domain

```
Human      MSVTEEDLCHHMKVVVRVRPENTKEKAAGFHKVVHVVDKHILVFDPKQEEVSFFHGKKTT 60
Zebrafish  --MSNGEVCSHVKVVVRVRPLNDKEKDGNFKKVVHVVDNHMLVFDPKEEEVTFFRGQRVG 58
             ::: ::* *:******** * *** ..*:*******:*:******:***:**:*::.

Human      NQNVIKKQNKDLKFVFDAVFDETSTQSEVFEHITKPILRSFLNGYNCTVLAYGATGAGKT 120
Zebrafish  NRDVRKRANKDLKFVFDSVFGEESSQIEVFENITKAIVDGVLNGYNCTVFAYGATGAGKT 118
           *::* *: *********:**.* *:* ****:***.*: ..*********:**********

Human      HTMLGSADEPGVMYLTMLHLYKCMDEIKEEKICSTAVSYLEVYNEQIRDLLVNSGPLAVR 180
Zebrafish  HTMLGTSNSPGVMFLTMKELFARMDLIKEDKVFNVAFSYLEVYNEQIRDLLTNSGPLAVR 178
           *****:::.****:*** .*:  ** ***:*: ..*.*************.********

Human      EDTQKGVVVHGLTLHQPKSSEEILHLLDNGNKNRTQHPTDMNATSSRSHAVFQIYLRQQD 240
Zebrafish  EDGSNGVVVQGLTLHQPKSAEHILEALDYGNRNRTQHPTDMNATSSRSHAVFQIYLRQQD 238
           ** .:****:*********:*.**. ** **:**************************

Human      KTASINQNVRIAKMSLIDLAGSERASTSGAKGTRFVEGTNINRSLLALGNVINALADSKR 300
Zebrafish  KTASLNFNVRVAKMSLIDLAGSERASATNTKGARLREGANINRSLLALGNVINTLANPKC 298
           ****:* ***:***************::.:**:*: **:**************:**:.*

Human      KNQHIPYRNSKLTRLLKDSLGGNCQTIMIAAVSPSSVFYDDTYNTLKYANRAKDIKSSLK 360
Zebrafish  KKTHIPYRDSKLTRLLKDSLGGNCRTVMIANVSPSSLSYEDTHNTLKYANRAKEIKSTLR 358
           *: *****:***************:*:*** *****: *:**:**********:***:*:

Human      SN 362
Zebrafish  SN 360
           **
```

Neck

```
Human      VLNVNNHITQVVKICNEQKAEILLLKEKLKAYEEQKAFTNENDQAKLMISNPQEKEIERF 60
Zebrafish  VMSLDSHIGQQYAIICEKQKAEIVMLKQKLKEYEERKAEAPALN----PISIQKRAEFEKM 56
           *:.::.** **. **::*****::**:*** ***:** :   :     **  :. *:*::

Human      QEILNCLFQNREEIRQEYLKLEMLLKENEL 90
Zebrafish  SESLRSVFSDRLKLRKEHLDIEKQLNESRL 86
           .* *..:*.:* ::*:*:*.:*  *:*..*
```

Stalk

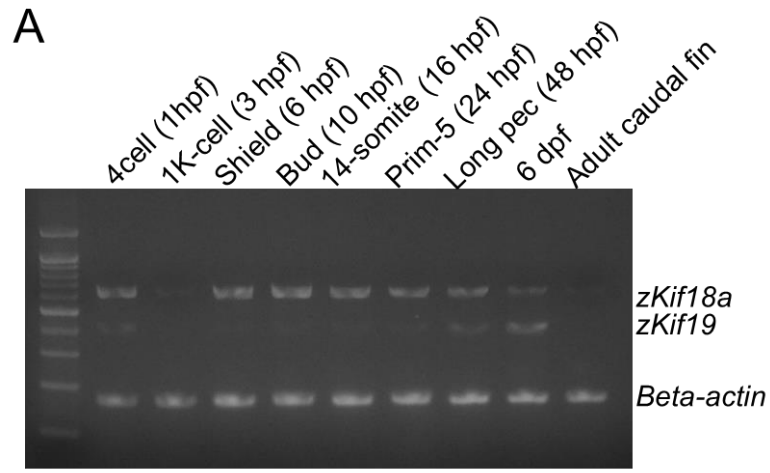
```
Human      KSFYQQQCHKQIEMMCSEDKVEKATGKRDHRLAMLKTRRSYLEKRREEELKQFDENTNWL 60
Zebrafish  TMRHRESWHQQSLIFFPDNKAERATCKYERKLASLKSHQEHLQQRLMESEKCFQENEGWL 60
           .  :::. *:*  :: .::*.*:** * :::** **:::.:*::*  *. * *:** .**

Human      HRVEKEMGLLSQNGHIPKELKKDLHCHHLHLQNKDLKAQIRHMMDLACLQEQQHRQTEAV 120
Zebrafish  HRIENEMKLLGHDGHTPEELKKELQCHQLQLQISDLQQHMEHMSHLISVQDQENTHTHKL 120
           **:*:** **.::** *:****:*:**:*:** .**: ::.** .* .:*:*:: :*. :

Human      LNALLPTLRKQYCTLKE 137
Zebrafish  VNALLSTCRRQHLALK- 136
           :****.* *:*: :**
```

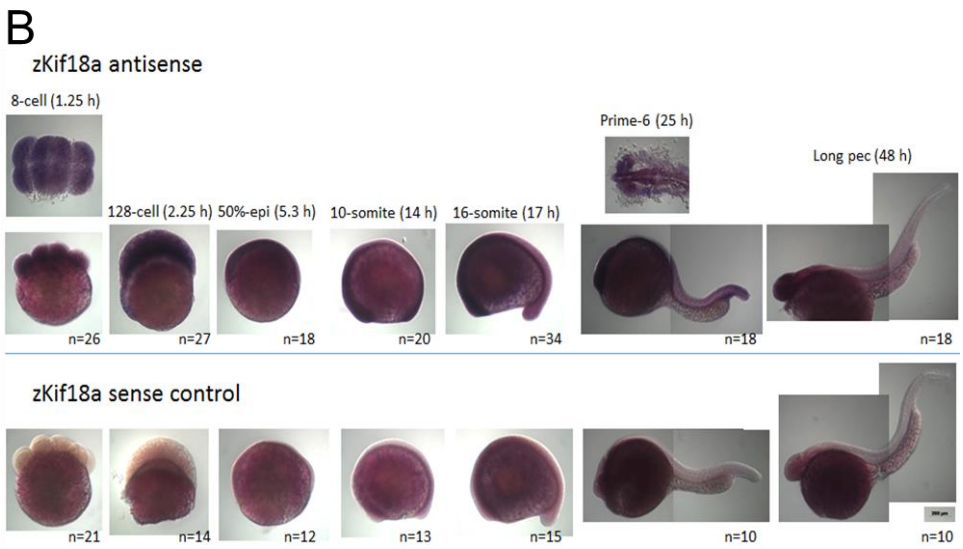
Tail

```
Human      AGLSNAAFESDFKEIEHLVERKKVVVWADQTAEQPKQND----LPGISVLMTFPQLGPVQ 56
Zebrafish  -AMSADGDGSEWEELERLVHRERAVVWADQEKAEDKRDDSGIGSTHLRPILSFSHLVSHQ 59
            .:*  .  *:::*:*:**.*::.******   : *::*     . :  :::*.:* . *

Human      PIPCCSS--SGGTNLVKIPTEKRTRRKLMPSPLKGQHTLKSPFSQSVQLN-DSLSKELQP 113
Zebrafish  STPCSSEKHTRRTSQRLRPTHSSKDCGPHHQVAEAEGVLKKPIRRKLSVSPAKLDGDGFV 119
           . **.*.  :  *.    **.. .      .  :.: .**.*  :.:.:.  .*. :

Human      IVYTPEDCRKAFQNPSTVTLMKPSSFTTSFQAISSNINSDNCLKMLCEVAIPHNRRKECG 173
Zebrafish  LSHHPPDLQDVLRQEG----MFPLQFTPEGQPHTSSIFDPNSTIDIGHIEDFTGANATII 175
           : : * * :..::: .    * * .**.. *. :*.* . *.   : .:  * . .

Human      QEDLDSTFTICEDIKSSKCKLPEQESLPNDNKDILQRLDPSSFSTKHSMFVPSMVPSYMA 233
Zebrafish  LSPEDPGRSTLQALRANNANKPAN-----------RLEIPSL-----LDLRRSKPSYMA 218
            .  *.  :  : :::.:.: * :           **: .*:     : :    *****

Human      MTTAAKRKRKLTSSTSNSSLTADVNSGFAKRVRQDNSSE------KHLQENKPTMEHKRN 287
Zebrafish  MTSAAQGKRKLNCSSIGKEDSSQAMAAAPKRIKRDPCEASKPLRIRRLGVSSENEPSRRV 278
           **:**: ****..*: ... :::. :. .**::* ..       ::*  .. .   :*

Human      ICKINPSMVRKFGRNISKGNLR---------- 309
Zebrafish  VRSVSEGNLHQLGRRKHKLSSMGPAALFKKVTKRM 313
           : .:. . ::::**.  * .
```

Figure 12. Kif18a mRNA expression pattern suggests its importance during embryogenesis. (A) Zebrafish has two kinesin-8 family proteins, Kif18a and Kif19. Kif18a and Kif19 mRNAs are both expressed as maternal and zygotic transcripts indicating its importance during embryogenesis. Maternal to zygotic transition (MZT) seems to occur at 3 hpf (hour post fertilization) for zKif18a, 3-6 hpf for zKif19. It may suggest that Kif18a is more important in early embryogenesis and Kif19 is taking charge of late embryogenesis. (B) *In situ* hybridization assay shows Kif18a mRNAs are mostly expressed where mitosis takes place.

Figure 12


A
4cell (1hpf)
1K-cell (3 hpf)
Shield (6 hpf)
Bud (10 hpf)
14-somite (16 hpf)
Prim-5 (24 hpf)
Long pec (48 hpf)
6 dpf
Adult caudal fin
zKif18a
zKif19
Beta-actin


B
zKif18a antisense
8-cell (1.25 h)
128-cell (2.25 h)
50%-epi (5.3 h)
10-somite (14 h)
16-somite (17 h)
Prime-6 (25 h)
Long pec (48 h)
n=26
n=27
n=18
n=20
n=34
n=18
n=18
zKif18a sense control
n=21
n=14
n=12
n=13
n=15
n=10
n=10

III-7. Zebrafish Kif18a is important during embryogenesis.

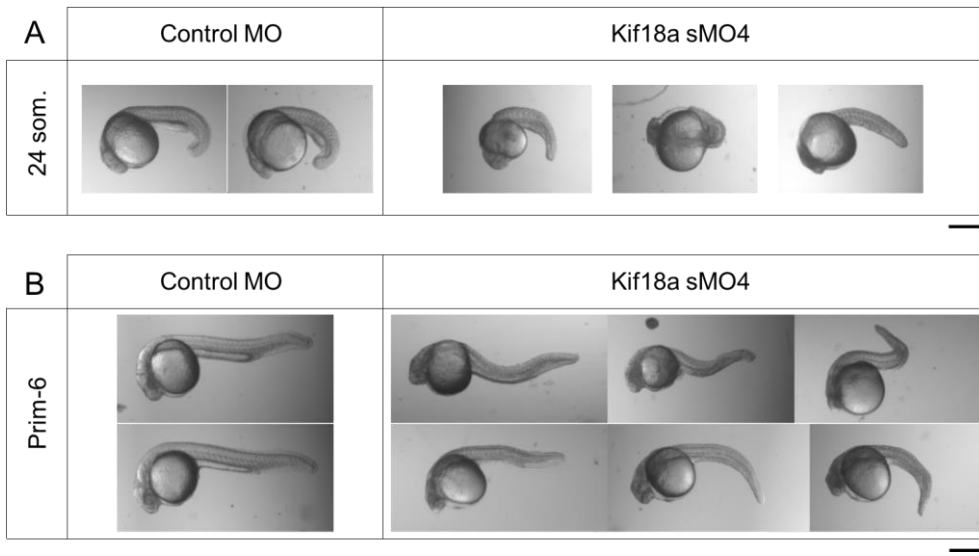
Kif18a expression pattern suggests its significance in zebrafish embryogenesis. To check whether Kif18a is essential or not, I injected Kif18a morpholinos. 1 pmole/embryos injection of Kif18a exon4-intron4 splicing blocking morpholinos induces growth defects after bud stages. As the maternal transcripts are not influenced by splicing blocking morpholinos, the morpholinos could be effective after the MZT (maternal to zygotic transition). In Kif18a morphants, skin epidermis was rough, head and brain region was smaller and yolk extension was not fully expanded and trunk was bended due to irregular cell expansion compared to the control (Figure 13). These phenotypes might be attributable to the mitotic failures during zebrafish embryogenesis, suggesting that Kif18a might have important roles in mitosis and development.

Collectively, the fact that Kif18a is conserved and the expression pattern and knockdown phenotypes of Kif18a indicates its importance during zebrafish embryogenesis, unlike mice.

Also, as the head and brain region, where Kif18a expressed, was defective upon Kif18a depletion in zebrafish, it could be an intriguing question to ask whether Kif18a is involved in neurodevelopment.

Figure 13. Kif18a knockdown induces growth defects. (A) Control morpholino and Kif18a exon4-intron4 splicing blocking morpholino were injected to 1-cell embryos and raised for the phenotype comparison. At 24 somite stage, living embryos were taken pictures. Kif18a morphant embryos were unhealthy and distorted compared to control. (B) 1 day after the morpholino injection. Kif18a morphants showed deaths in head region and bended trunks. Embryos for control and Kif18a splicing block morpholino injected were pictured under the stereomicroscopy (Zeiss, Lumar.V12 steREO). Scale bar, 500 μm.

Figure 13


A
Control MO
Kif18a sMO4
24 som.
B
Control MO
Kif18a sMO4
Prim-6

III-8. Zebrafish Kif18a C-terminal domain is dispensable for viability and fertility

Morpholino injection technique is still working, but there could be a toxicity in itself such as necrosis in brain and edema around yolk. So, it is recently suggested to use knockout or mutant fish to support and confirm the precise morpholino phenotypes.

To find the role of Kif18a in zebrafish embryogenesis, Kif18a knockout fish is generated using ZFN (zinc finger nuclease) targeting exon 8 region. As exon 8 spans for the neck domain and 5 nucleotides deletion in this region makes frameshift leading to premature stop codon, mutant fish is thought to express the truncated proteins which have intact motor domain but lack C-terminus (Figure 14). I reasoned that it could provide the information on not only the physiological roles of Kif18a, but also whether depolymerase activities in C-terminus are important or not.

Unexpectedly and interestingly, the mutant fish were existed in the

intercross of heterozygous mutant as mendelian ratio (Homozygous : Heterozygous : Wildtype is approximately 1:2:1).

Furthermore, the homozygous mutants were viable to grow into adult fish and fertile to have enough large clutch without any gender bias (Figure 15).

Germ-line transmission of the mutation and viability of homozygous mutant were confirmed by sequencing. Homozygous mutant fishes were intercrossed and their eggs were harvested. mRNAs from the egges were isolated with Trizol and were reverse-transcribed. The target mutated region was amplified with PCR from the cDNAs and then the PCR products were cloned into TOPO vectors. All sequenced clones showed 5 nucleotides deletion in targeted region of Kif18a, indicating the homozygous mutant fish were viable and the mutation transmitted to progenies. (Figure 16).

So, I could conclude that the C-terminal domain of Kif18a is dispensable for viability and fertility, demonstrating the motor domain of Kif18a is merely important and functional part in zebrafish.

Figure 14. Kif18a C-terminal domain disrupted zebrafish is generated using ZFN (zinc finger nuclease) technology. (A) Targeted disruption of Kif18a in zebrafish is performed by ZFN (zinc finger nuclease) technology. Targeting region in exon 8 is marked in a yellow shaded box. After the genome editing by ZFN, 5 nucleotides are deleted from 4 in targeting region and 1 in right arm. (B) 5 nucleotides deletion in exon 8 makes frameshift leading to premature stop codon. Therefore, mutant zebrafish is thought to express the truncated proteins which have intact motor domain but lack C-terminus. A putative expressed amino acid sequence and protein are drawn.

Figure 14

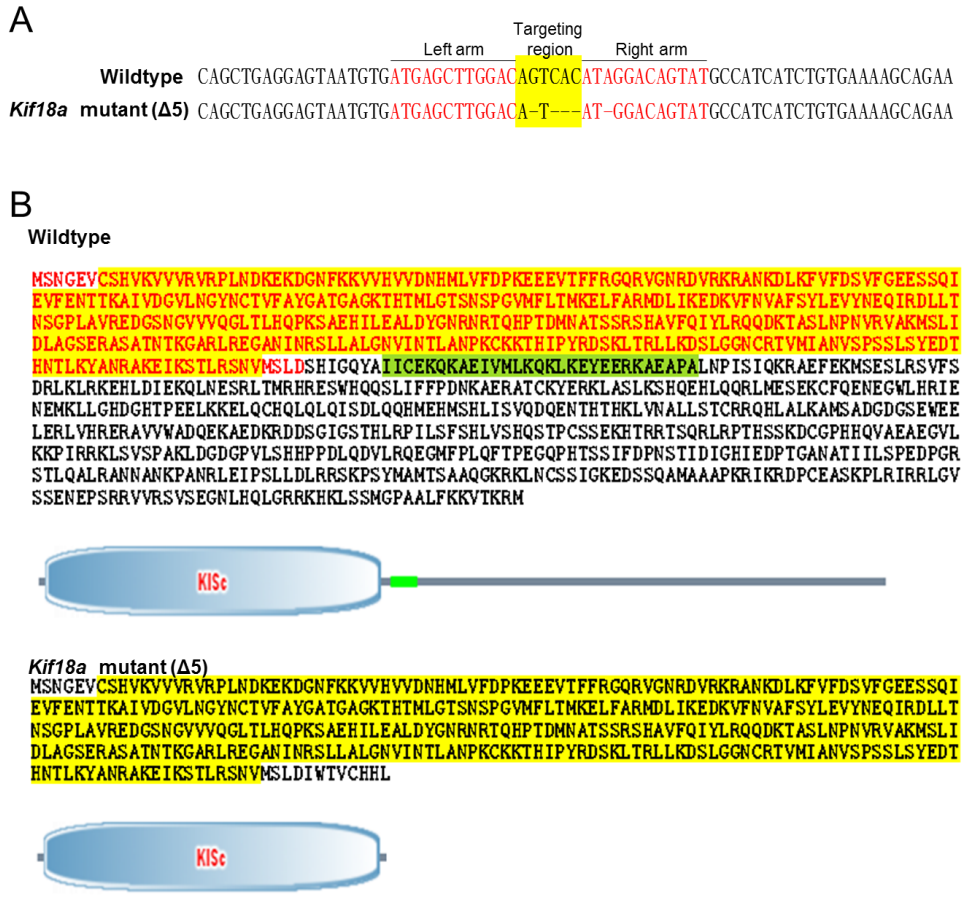

A
Left arm
Targeting region
Right arm
Wildtype CAGCTGAGGAGTAATGTGATGAGCTTGGACAGTCACATAGGACAGTATGCCATCATCTGTGAAAAGCAGAA
Kif18a mutant (Δ5) CAGCTGAGGAGTAATGTGATGAGCTTGGACA-T---AT-GGACAGTATGCCATCATCTGTGAAAAGCAGAA
B
Wildtype
MSNGEVCSHVKVVVRVRPLNDKEKDGNFKKVVHVVDNHMLVFDPKEEEVTFFRGQRVGNRDVRKRANKDLKFVFDSVFGEESSQI
EVFENTTKAIVDGVLNGYNCTVFAYGATGAGKTHTMLGTSNSPGVMFLTMKELFARMDLIKEDKVFNVAFSYLEVYNEQIRDLLT
NSGPLAVREDGSNGVVVQGLTLHQPKSAEHILEALDYGNRNRTQHPTDMNATSSRSHAVFQIYLRQQDKTASLNPNVRVAKMSLI
DLAGSERASATNTKGARLREGANINRSLLALGNVINTLANPKCKKTHIPYRDSKLTRLLKDSLGGNCRTVMIANVSPSSLSYEDT
HNTLKYANRAKEIKSTLRSNVMSLDSHIGQYAIICEKQKAEIVMLKQKLKEYEERKAEAPALNPISIQKRAEFEKMSESLRSVFS
DRLKLRKEHLDIEKQLNESRLTMRHRESWHQQSLIFFPDNKAERATCKYERKLASLKSHQEHLQQRLMESEKCFQENEGWLHRIE
NEMKLLGHDGHTPEELKKELQCHQLQLQISDLQQHMEHMSHLISVQDQENTHTHKLVNALLSTCRRQHLALKAMSADGDGSEWEE
LERLVHRERAVVWADQEKAEDKRDDSGIGSTHLRPILSFSHLVSHQSTPCSSEKHTRRTSQRLRPTHSSKDCGPHHQVAEAEGVL
KKPIRRKLSVSPAKLDGDGPVLSHHPPDLQDVLRQEGMFPLQFTPEGQPHTSSIFDPNSTIDIGHIEDPTGANATIILSPEDPGR
STLQALRANNANKPANRLEIPSLLDLRRSKPSYMAMTSAAQGKRKLNCSSIGKEDSSQAMAAAPKRIKRDPCEASKPLRIRRLGV
SSENEPSRRVVRSVSEGNLHQLGRRKHKLSSMGPAALFKKVTKRM
KISc
Kif18a mutant (Δ5)
MSNGEVCSHVKVVVRVRPLNDKEKDGNFKKVVHVVDNHMLVFDPKEEEVTFFRGQRVGNRDVRKRANKDLKFVFDSVFGEESSQI
EVFENTTKAIVDGVLNGYNCTVFAYGATGAGKTHTMLGTSNSPGVMFLTMKELFARMDLIKEDKVFNVAFSYLEVYNEQIRDLLT
NSGPLAVREDGSNGVVVQGLTLHQPKSAEHILEALDYGNRNRTQHPTDMNATSSRSHAVFQIYLRQQDKTASLNPNVRVAKMSLI
DLAGSERASATNTKGARLREGANINRSLLALGNVINTLANPKCKKTHIPYRDSKLTRLLKDSLGGNCRTVMIANVSPSSLSYEDT
HNTLKYANRAKEIKSTLRSNVMSLDIWTVCHHL
KISc

Figure 15. C-terminal domain of zebrafish Kif18a is dispensable for viability and fertility. (A) Intercross of Kif18a heterozygous mutant shows offspring with mendelian ratios. (Homozygous:Heterozygous:WT=4:8:4). Representative genotyping was performed with F3 progenies (Date of birth: 29th Apr 2014). (B) Homozygous mutant zebrafish can grow and lay eggs with homozygous genotype. More than two independent pair of homozygous mutants were tested for fertility.

Figure 15

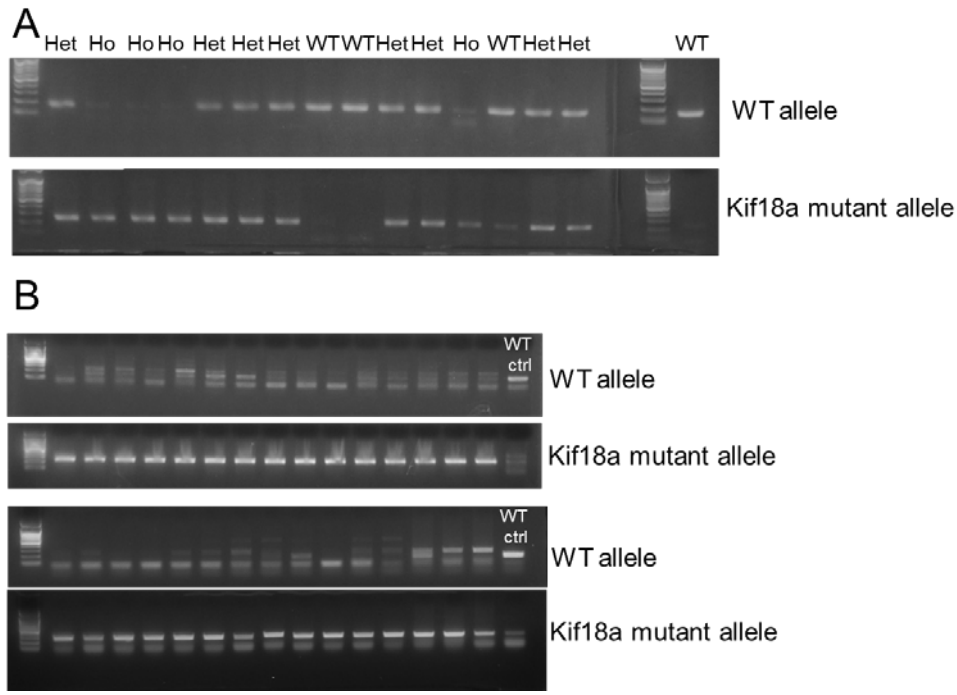
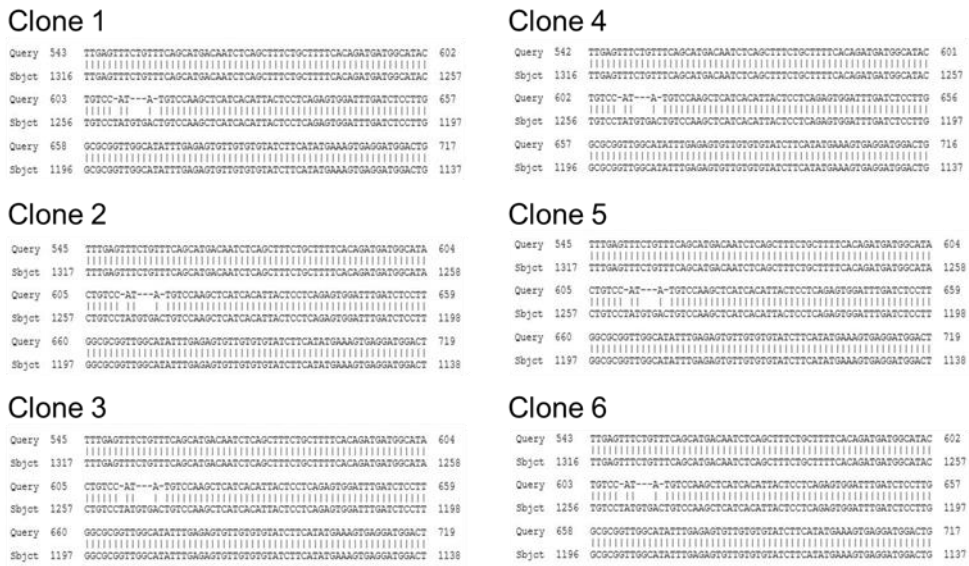

A
Het Ho Ho Ho Het Het Het WT WT Het Het Ho WT Het Het
WT
WT allele
Kif18a mutant allele
B
WT ctrl
WT allele
Kif18a mutant allele
WT ctrl
WT allele
Kif18a mutant allele

Figure 16. Eggs from intercrossed homozygous mutants have 5 nucleotides deletion in targeted region of Kif18a. Homozygous mutant fishes were intercrossed and their eggs were harvested. mRNAs from the egges were isolated with Trizol (Invitrogen) and were reverse-transcribed with RTaseII (Invitrogen). The targeting region was amplified with PCR from the cDNAs and then the PCR products were cloned into TOPO vector (Invitrogen). All sequenced clones showed 5 nucleotides deletion in targeted region of Kif18a, indicating the homozygous mutant fish were viable and the mutation transmitted to progenies.

Figure 16



III-9. Kif18a C-terminal domain is not required for proper chromosome segregation

As Kif18a C-terminal domain is dispensable for viability and fertility of zebrafish, it implies that motor domain, not C-terminus, is actually functional in physiology. If so, I wondered if the C-terminus is not important for mitosis. To analyze the ZFN mutant phenotype deeply, I observed the cell division in mutant fish.

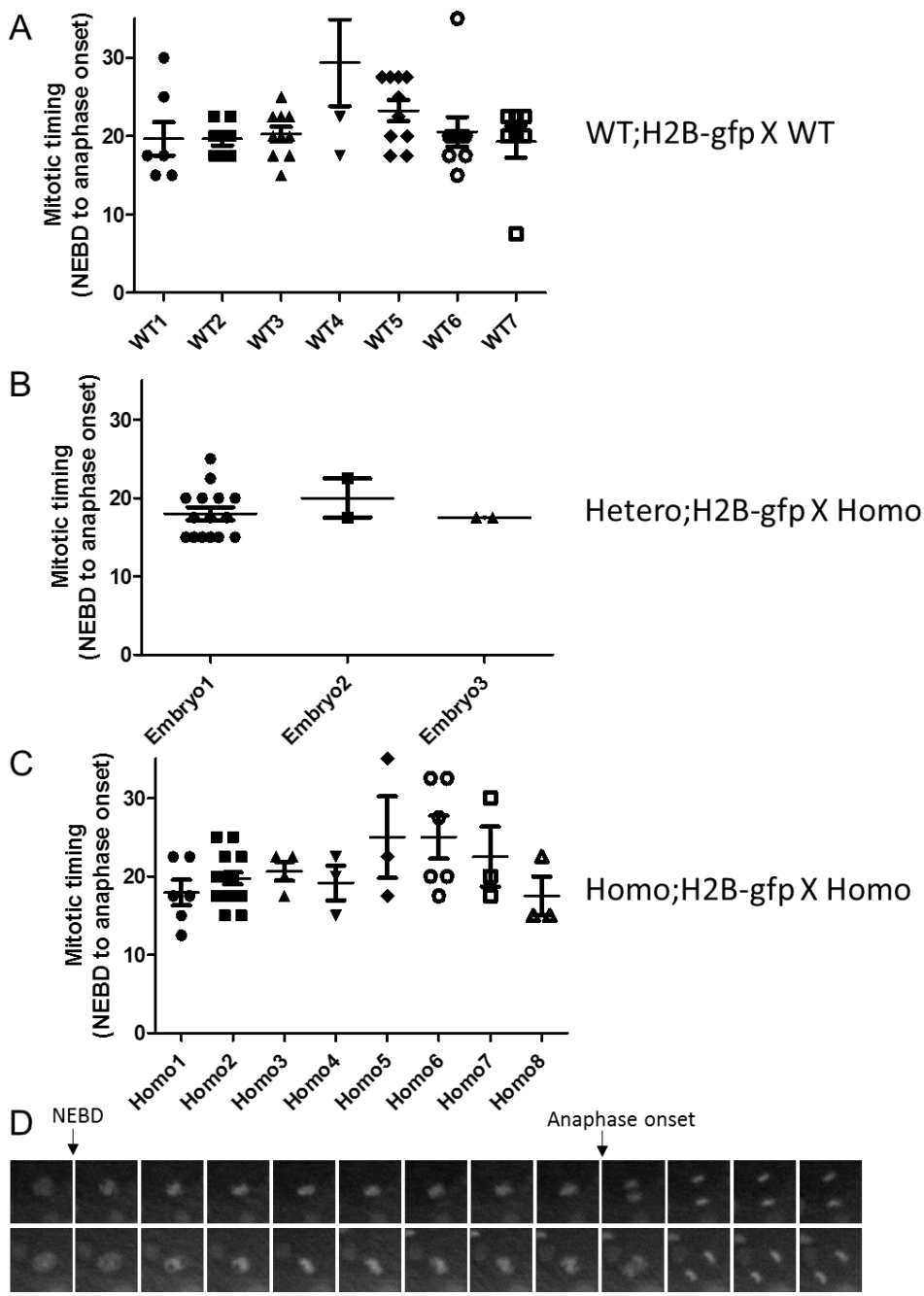
First, heterozygous mutants expressing H2B-gfp is crossed with homozygous mutant fish. And I selected GFP expressing embryos that might have 1 to 1 Homozygous:Heterozygous ratios and live-imaged for 24 hours. By stochastical analysis from obtained data from 3 embryos at once, there is no change in mitotic timing and chromosome movement.

Moreover, homozygous mutant embryos expressing H2B-GFP showed no significant change in mitotic timing and chromosome segregation. Mitotic timing is measured by the duration from NEBD (nuclear envelope breakdown)

to anaphase onset and scattered plot is drawn by GraphPad Prism software. As shown, mitotic timing were 15–20 minutes in cells from both control and Kif18a mutant embryos. I could conclude that the C-terminal domain of Kif18a is not required for the mitotic timing and chromosome segregation. This results provoke that Kif18a motor domain, not C-terminus, is genuine functional domain for Kif18a roles, dampening chromosome oscillation and regulating mitosis.

Figure 17. The C-terminal domain of Kif18a is not required for chromosome segregation. (A) Wild type embryos expressing H2B-gfp show normal cell division. Normal mitotic timing from NEBD (nuclear envelope breakdown) to Anaphase onset takes 15-20 minutes in zebrafish embryogenesis. (B) Heterozygous mutants expressing H2B-gfp is crossed with homozygous mutant fish and mitotic timing from 3 GFP expressing embryos were measured. Stochastically, these embryos can have 1/2 homozygous and 1/2 heterozygous genotypes. There is no significant change in mitotic timing and chromosome movement. (C) Homozygous mutant embryos expressing H2B-GFP showed relatively normal mitotic timing for 15-20 minutes. (D) Representative cells from (C). Images are taken as 2.5 minute intervals with DeltaVision (100X objectives) and processed with Image J.

Figure 17


A
Mitotic timing
(NEBD to anaphase onset)
30
20
10
0
WT1
WT2
WT3
WT4
WT5
WT6
WT7
WT;H2B-gfp X WT
B
Mitotic timing
(NEBD to anaphase onset)
30
20
10
0
Embryo1
Embryo2
Embryo3
Hetero;H2B-gfp X Homo
C
Mitotic timing
(NEBD to anaphase onset)
30
20
10
0
Homo1
Homo2
Homo3
Homo4
Homo5
Homo6
Homo7
Homo8
Homo;H2B-gfp X Homo
D
NEBD
Anaphase onset

IV. DISCUSSION

IV-1. Zebrafish, as a model system to study mitosis and development, mirroring tumorigenesis

These results not only discover the function of Aurora-A and Kif18a in zebrafish, but also have beneficial implications. It has been recognized that zebrafish as a cancer model (Feitsma and Cuppen, 2008) and cell cycle regulators as an important factor for development (Budirahardja and Gonczy, 2009). I propose that zebrafish embryogenesis is likely to reflect the cell proliferation of tumorigenesis, since rapid cell division cycle is one of the characteristics for the duration of embryogenesis. Despite the existing limitations, it could be capable of adopting zebrafish embryos as a stages of *in vivo* drug discovery (Zon and Peterson, 2005) small molecule screening (Murphey and Zon, 2006) or drug validation (Jeong et al., 2010).

IV-2. Safeguarding of genomic integrity in normal embryogenesis

I showed that abnormal spindle formation and defective mitotic progression occurred in the absence of Aurora-A. Nevertheless, when chromosomes oscillate back and forth near the equatorial plate, cells might have enough time to correct spindle-kinetochore attachment. Thus, the cells would be able to form bipolar spindles, congress chromosomes, and satisfy the spindle assembly checkpoint, which would result in successful cell division with minor segregation errors. However, small portion of cells bearing severe chromosome segregation errors might be largely affected, succumbing to apoptosis at post-mitotic G1 phase in p53-dependent manner. These processes imply the possibility that safeguarding mechanisms preserving genomic integrity prevent tumorigenesis and maintain homeostasis in the setting of normal embryogenesis.

IV-3. The functions of Aurora-A in zebrafish system

It is quite intriguing that the known function of Aurora kinase A is mainly on the centrosome and spindle formation, but has controversies depending on the species and the system. To grasp the actual, ancient and conserved Aurora-A function and to reach out consent, the study digging up Aurora-A function using another species or systems is necessary. Additionally, to detour the embryonic lethality of Aurora-A knockout mice, our morphant zebrafish embryogenesis is proficient *in vivo* system. I noticed that the conserved zebrafish Aurora-A was expressed during embryogenesis and Aurora-A morphants displayed developmental defects. This insinuates that Aurora-A has its roles in development and proliferation. Besides, from my results, growth abnormalities might have been affected by mitotic progression defects, induced by monopolar spindles and delayed mitotic timing, and by cell death. It seems that the roles of Aurora-A in mitotic progression and cell death are preserved through the species. On the other hand, another report suggests that Aurora-A depletion by antibody injection induces multipolar spindles and

multinucleated cells in cultured HeLa cell-line (Marumoto et al., 2003). Antibody injection has advantages, such as dissecting cell cycle-dependent function by determining the injection time point. However, the discrepancy with our results might be attributable to the epitope issues, that is, antibodies are only able to block the function of the antibody binding site, N-terminal domain in this case.

IV-4. Underlying molecular mechanism of anti-cancer drug targeting Aurora-A

As for the cancer therapy, tailored medicine could be the ultimate goal. Conventionally, the most popular way to cure cancer is the use of chemotherapeutic treatment of taxane agents or platinum. However, resistance to chemotherapy is becoming hard to tackle for the anti-cancer therapeutics. To solve this problem, efforts to develop and clinically applicate anti-mitotic drugs have been receiving much attention recently. Among

Aurora-A inhibitors, MLN8237 (alisertib) is currently under evaluation in a phase I/II trials (Cervantes et al., 2012; Dees et al., 2012; Matulonis et al., 2012). It is also reported that established chemotherapy drug resistance tends to be higher in p53-positive cancer (Anelli et al., 2003). Therefore, Aurora-A inhibitors targeting proliferating cancer cells could be the alternates that bypass the taxol- and platinum-resistance.

Although the clinical data are accumulating, the underlying molecular mechanism of treatment of anti-cancer drugs that target Aurora-A is largely unknown. In this respect, our results using the zebrafish system could provide the molecular mechanism of cancer cell death with the Aurora-A inhibition. As mentioned earlier, rapid cell cycle progression within the zebrafish embryogenesis resembles cancer cell proliferation. I discovered that Aurora-A depletion induces p53-dependent apoptosis in our system. It is reported that regulating of Mdm-2 degradation by Aurora-A eventually destabilize p53 (Katayama et al., 2004). Taken together, in addition to explaining the high correlation between Aurora-A and p53, I could speculate that the Aurora-A

inhibitors are more effective to chemotherapy-resistant, p53-positive cancer patients, with our results. This work will give personalized medicine according to p53 status a molecular-base mechanism. Such studies revealing underlying mechanisms of anti-cancer therapeutics should be further needed.

IV-5. Dissecting the roles between N-terminal Motor and C-terminus of Kif18a

Kif18a is a kinesin-8 family protein known to have N-terminal motor and C-terminal depolymerase domain. I suggests that the role of Kif18a in zebrafish can be dissected using two kinds of knockout fish. As Kif18a C-terminal domain is not required for the viability, fertility, and mitosis, I am now generating Kif18a motor domain targeted knockout zebrafish using CRISPR/Cas9 technique (Fig 18). By adopting the 2 vector system (Hwang et al., 2013), Cas9 mRNA and guide RNA were synthesized from these vector, respectively. And I found that the exon 3 targeted guide RNAs have ability to genome editing. As Kif18a might have roles in mitosis and development in

zebrafish according to the expression pattern and knockdown phenotypes, this knockout zebrafish will contribute in defining the physiological role of Kif18a.

IV-6. Implications for the conserved roles of Kinesin-8 family proteins

As Kif18a morphants exhibited growth abnormalities and small heads, it implicates that the Kif18a might have its role in mitosis and brain development. However, the fact that Kif18a knockout/mutant mice disrupted motor domain only showed male infertility due to mitotic infidelity suggests that there is still limited knowledge on the physiological role of Kif18a. It reminiscent that it is possible that motor targeted knockout fish do not show significant phenotypes. If so, there might be a compensation mechanism *in vivo*. I suggest that Kif19, the other kinesin-8 family member, is one of the candidates. Kif18a and Kif19 both expressed as maternal and zygotic transcripts, but after the MZT, Kif18a is gradually diminished and Kif19 is gradually increased as they grow (Figure 12), suggesting compensational

exist. In fact, knockout mice for Kif19 showed hydrocephalus and female infertility phenotypes due to abnormally elongated cilia that cannot generate proper fluid flow (Niwa et al., 2012). It is quite interesting that Kif18a knockout mice show male infertility and Kif19 knockout mice show female infertility. Since there are only two known kinesin-8 family proteins in zebrafish, Kif18a and Kif19, generation of double knockout fish could help to understand kinesin-8 biology.

IV-7. Perspectives in cancer therapy

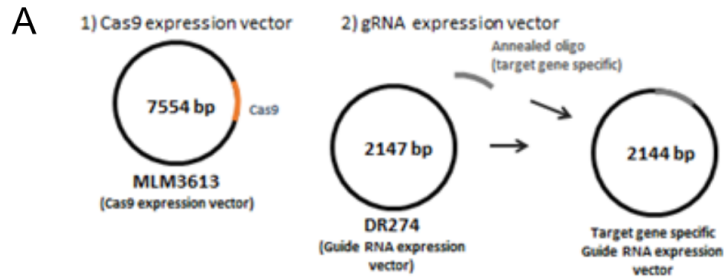
Currently, cancer is known to be not only a disease of genomic instability, but more complicated. Although cancer incidences increase every year due to many reasons such as longevity, early detection, environmental effects, tobacco smoking, it seems to turn out that “the war on cancer” has not been won and there is no magic bullet for cancer therapies. Even though there are lager collected information on cancer hallmarks, cancer genomes and carcinogenesis mechanisms from academia, hospitals, governments, cancer is

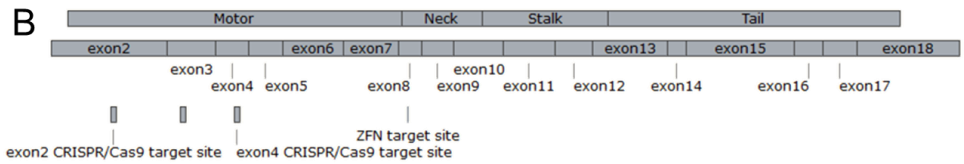
too sophisticated that the curation of cancer is still incomplete depending on the diagnosed stages, types of cancer, arisen tissues, invasiveness/metastasis, etc. Moreover, anti-cancer drugs are effective only for small subsets of patients and even worse, constant treatment of them can induce resistance to chemotherapy, leading to more aggressive, invasive and metastasis cancers that is harder to remedy. A recent review by Douglas Hanahan describes well about the contemporary situation (Hanahan, 2014). In this series of reviews, it is suggested that there are many aspects of rethinking, for example, cancer prevention, cultural and economic view and cancer therapy when dealing with cancer. Especially for the cancer therapy, three perspectives of battlefields are suggested to be redefined to fight for the cancer. The first is about co-targeting cancer hallmarks. There are typical cancer hallmarks showing many capabilities of cancer, for example, evading from growth suppressors and continuously dividing, avoiding from cell death and activating invasiveness/metastasis. Concomitant disrupting for two or more of those cancer capabilities could be more effective strategies. The second part is targeting surroundings of cancer besides cancer cell itself. Stromal cells that

support cancer growths, vasculature devices and cancer stem cells could be. The third is considering the tumor microenvironments along cancer stages, as they are different between primary tumors and aggressive/invasive tumors. As putting into the suggested considerations, there are more needs of preclinical models of human cancer and zebrafish embryogenesis could be one of them. I showed, using zebrafish embryogenesis, that the inhibition of Aurora-A could be only effective to p53-positive cells, and this somewhat disappointing result still gives us information in redefined strategies for cancer therapeutics. We should be cautious to apply Aurora-A inhibitors to patients; early detection might be required when p53 should not be mutated and p53 status might be reflected for decision making of drug treatment for personalized therapy. Although this study is restricted to Aurora-A and Kif18a, further use of zebrafish embryogenesis in studying mitosis and cancer could provide more knowledge on pre-clinical results that support the baseline mechanism of cancer treatment.

Figure 18. Schematic drawing for strategy to generate Kif18a motor domain deletion mutant using CRISPR/Cas9 technology. (A) Two vector system adopted: 1) Cas9 expression vector (MLM3613) and 2) guide RNA expression vector (DR274). Cas9 mRNA and guide RNAs are linearized and synthesized *in vitro* using T7 RNA polymerase. (B) Exons in Kif18a. (C) For targeted disruption in Kif18a motor domain, exon 2, exon 3, exon 4 targeted guide RNAs are generated. Targeted regions are selected for having minimum potential off-targets in genome using ZiFiT Targeter supported by zinc finger consortium (http://zifit.partners.org/ZiFiT/). A pair of phosphorylated oligos for target sites is annealed and ligated with Bsa1-digested DR274 vector. (D) Exon 3 targeted guide RNA worked for genome editing. Exon 3 guide RNA and Cas9 mRNA were injected to 1-cell embryos and then raised. Grown-up F0 founder fishes were screened with fin clip DNA by T7E1 (T7 endonuclease 1) assay. As T7E1 cleaves mismatched double strand DNA, fish number 5 is thought to be a genome-edited heterozygous mutant.

Figure 18

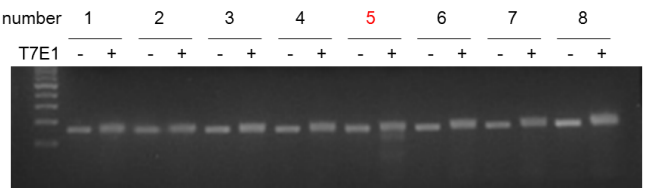




C

targeted exon	Target site	Oligo 1	Oligo 2	potential offtarget
exon2	GGAAGAAGTGACATTTTTCC	TAGGAAGAAGTGACATTTTTCC	AAACGGAAAAATGTCACTTCTT	1
exon3 – Reverse Strand	GGAATTACTGGTGCCCAACA	TAGGAATTACTGGTGCCCAACA	AAACTGTTGGGCACCAGTAATT	no offtarget
exon4	GGACGGCTCCAATGGAGTTG	TAGGACGGCTCCAATGGAGTTG	AAACCAACTCCATTGGAGCCGT	2

D



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국문 초록

세포 분열의 꽃은 mitosis 시기이다. 이 때 복제된 염색체가 두 딸세포로 균등하게 갈라짐으로써 유전체 안정성이 유지될 수 있는데, 이를 정교하게 조절하기 위해 다양한 mitotic kinase, phosphatase, kinesin들이 mitosis에 참여한다. 본 논문은 zebrafish embryogenesis를 모델로 하여 mitotic kinase, mitotic motor protein과 같은 mitotic machinery를 연구하였다. Species 간에 잘 보존되어 있는 기능이 가장 본연의 것이라는 전제 하에, 척추동물인 zebrafish에서 mitotic machinery들의 기능을 규명함으로써, 세포 분열기에 대한 학문적 이해를 높이고자 하였다. 기존의 세포주 실험이나 생화학적 기법에 의존한 연구들은 생체 내에서의 현상을 반영하기 어려운 한계를 지니고, 중요한 유전자들에 대한 knockout mouse는 초기 치사 빈도가 높으며, 종 (species) 별로 서로 다른 결과들을 보여왔다. 따라서, zebrafish embryogenesis를 모델로 세포분열 기작 연구를 진행한다면 이를 극복하고 진화적으로 보존된 세포분열기작을 규명할 수 있을 것으로 보인다. 여러 mitotic machinery 중 Aurora-A mitotic kinase와 Kif18a motor protein를 중심으로 연구를 진행하였는데, Aurora-A와 Kif18a의 기능 연구에 있어 기존의 세포주나 마우스 연구에서 불일치하는 결과 (discrepancy)를 해소하고 세포분열기작에 대한 이해를 고취하는 데 기여하고자 한다.

Zebrafish에서 Aurora-A가 잘 보존되어 있음을 확인하고, morpholino를 이용하여 Aurora-A knockdown 시 개체와 세포 수준의 phenotype을 관찰하였다. 그 결과 Aurora-A가 centrosome intactness에 중요하며, 이는 mitotic progression에 중요함을 밝혔다. 또한 Aurora-A 저해 시 p53 의존적 세포사멸이 일어난다는 것을 밝혀, 항암 타깃 전략 수립 시 활용 가능한 분자생물학적 단초를 제공할 수 있을 것으로 보인다. 이러한 기초 연구를 토대로 항암 치료 시 Aurora-A 저해제 투여가 효과적일 수 있을만한 암종을 선정하는 데 p53 status를 확인하는 등 일종의 기준을 제시할 수 있을 것으로 여겨진다. Kinesin-8 family motor protein인 Kif18a가 zebrafish 에서 잘 보존되어 있음을 확인하고, embryogenesis 중 Kif18a의 발현양상과 Kif18a morpholino 주입 시 보이는 developmental defect를 통해 Kif18a가 발생과정에 중요할 것이라는 단서를 얻었다. N-terminal motor domain의 motor activity와 C-terminal tail의 depolymerase activity 중 어느 것이 functional majority가 있는지 규명하기 위해, neck domain을 target한 ZFN knockout zebrafish를 제작하여 표현형을 분석하였다. 그 결과 Kif18a C-terminal domain은 개체의 생존 및 생식에 중요하지 않으며, 세포 분열 시간과 염색체의 움직임에도 중요하게 작용하지 않음을 밝혔다. 본 논문에서는 Aurora-A와 Kif18a에 한정하여 세포분열 기작 연구를 진행하였으나, 추후에도 zebrafish embryogenesis를 모델로 활용하였을 때의 장점과 유전학적 용이함을 십분 활용한다면 척추동물의 체세포분열에 대한 생물학적 제

문제를 해결할 수 있는 훌륭한 연구 성과들이 도출될 수 있을 것으로 기대된다.

주요어 : 제브라피시, 배발생과정, 세포분열기구, Aurora-A 키나아제, Kif18a 모터 단백질

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